Cardiovascular Effects of Environmental Tobacco Smoke and Benzo[a]pyrene Exposure in Rats

A thesis submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Toxicology Graduate Program University of Saskatchewan Saskatoon, Saskatchewan Canada

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PREFACE

Chapters 2, 3, and 4 of this thesis are organized as manuscripts that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material is unavoidable.

ABSTRACT

Smoking and environmental tobacco smoke (ETS) exposure are major risk factors for cardiovascular disease (CVD), although the exact components and pathophysiological mechanisms responsible for this association remain unclear. Polycyclic aromatic hydrocarbons (PAHs), including benzo[a]pyrene (BaP), are ubiquitous environmental contaminants that form during organic material combustion and are thus found in cigarette smoke, vehicle exhaust particles, and air pollution. We hypothesize that PAHs are key agents responsible for mediating the cigarette smoke effects in the cardiovascular system, including increased oxidative stress, inflammation, and arterial stiffness.

Arterial stiffness is a powerful, independent predictor of cardiovascular risk and is regulated, in part, by vasoactive mediators derived from the endothelium. The first objective of this project was to determine whether pulse wave dP/dt collected from radiotelemetry-implanted rats is a reliable indicator of changes in arterial stiffness following administration of vasoactive drugs or acute ETS exposure. Anaesthetized rats were administered a single dose of saline (vehicle control), acetylcholine, norepinephrine, and N(G)-nitro-L-arginine methyl ester (L-NAME) via the tail vein, allowing a washout period between injections. Acetylcholine decreased and norepinephrine increased dP/dt compared to saline vehicle. Injection of the nitric oxide (NO) synthase inhibitor L-NAME decreased plasma nitrate/nitrite (NOx), but transiently increased dP/dt. For the ETS experiment, rats were exposed for one hour to sham, low dose ETS, or high dose ETS. Exposure to ETS did not significantly alter dP/dt or plasma endothelin-1 (ET-1) levels, but increased plasma NOx levels at the high ETS exposure and increased plasma nitrotyrosine levels in both ETS groups. In conclusion, acute changes in NO production

via acetylcholine or L-NAME alter the arterial pulse wave dP/dt consistently with the predicted changes in arterial stiffness. Although acute ETS appears to biologically inactivate NO, a concomitant increase in NO production at the high ETS exposure may explain why ETS did not acutely alter dP/dt.

The second objective of this project was to compare the effects of subchronic ETS and BaP exposure on circadian blood pressure patterns, arterial stiffness, and possible sources of oxidative stress in radiotelemetry-implanted rats. Pulse wave dP/dt was used as an indicator of arterial stiffness, and was compared to both structural (wall thickness) and functional (NO production and bioactivity, ET-1 levels) features of the arterial wall. In addition, histology of lung, heart, and liver were examined as well as pulmonary and hepatic detoxifying enzyme activity (cytochrome P450 – specifically CYP1A1). Daily ETS exposure for 28 days altered the circadian pattern of heart rate and blood pressure in rats, with a loss in the normal dipping pattern of blood pressure during sleep. Subchronic ETS exposure also increased dP/dt in the absence of any structural modifications in the arterial wall. Although NO production and ET-1 levels were not altered by ETS, there was increased biological inactivation of NO via peroxynitrite production (as indicated by increased plasma nitrotyrosine levels). Thus, vascular stiffness and failure of blood pressure to dip precede structural changes in rats exposed to ETS for 28 days. Exposure to ETS also caused increased number of lung neutrophils as well as increased CYP1A1 activity in lung microsomes.

Since ETS-induced increases in arterial stiffness occurred as early as day 7, radiotelemetry-implanted rats were exposed daily to intranasal BaP for 7 days. Similar to ETS, BaP exposure altered circadian blood pressure patterns and reduced blood pressure

dipping during sleep. Thus, in support of part of our hypothesis, the PAH component of cigarette smoke may be responsible for the ETS-induced increase in blood pressure and the loss of dipping pattern during sleep. Increased neutrophil recruitment was observed in the lungs of both ETS- and BaP-exposed rats, suggesting that lung inflammatory reactions may be involved in the disruption of circadian blood pressure rhythms. Unlike ETS however, BaP exposure did not significantly alter pulse wave dP/dt, endothelial function, or lung CYP1A1 activity. Thus, contrary to our hypothesis, the reduction in NO bioactivity and increased arterial stiffness caused by ETS cannot be explained by BaP at the dose and length of the exposure in the current study. Production of reactive metabolites in the lung following ETS exposure may be responsible, at least in part, for the increases in oxidative stress in the vasculature, leading to reduced NO bioactivity and increased arterial stiffness. Oxidative stress caused by BaP exposure may have been insufficient to reduce NO bioactivity in the peripheral vasculature. Therefore arterial stiffness was not increased and factors other than NO may be responsible for the increase in blood pressure observed with ETS and BaP exposure.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Lynn Weber for her guidance and encouragement throughout this project. I would also like to thank the other members of my advisory committee, Dr. Linda Hiebert, Dr. Michael Pietrock, and Dr. Barry Blakley for assisting throughout the completion of this thesis and for providing various aspects of scientific expertise.

This thesis is dedicated to my family for all their love and support, including my husband Chad, my parents Brian and Darlene, my siblings Jaret and Breanne, and my friend Chelene. My gratitude and heartfelt thanks also go out to the members of the Weber laboratory as well as fellow students in the toxicology program, including Jordan Marit, Ahmad Aldissi, Brandie Bugiak, Meghan Goertzen, Jennifer Adolphe, Lilani Munasinghe, and Jonathan Naile. Finally, I would like to thank everyone at the Animal Care Unit as well as my friends and colleagues in the Toxicology Centre and the Department of Veterinary Biomedical Sciences.

Funding for this project was provided by the Natural Sciences and Engineering Research Council (NSERC) and the Saskatchewan Health Research Foundation (SHRF).

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LIST OF ABBREVIATIONS

ACh – acetylcholine

AhR - aryl hydrocarbon receptor

ANCOVA - analysis of covariance

AMI - acute myocardial infarction

Ang II - angiotensin II

ANOVA - analysis of variance

ARNT - AhR nuclear translocator

BaP - benzo[a]pyrene

bHLH/PAS - basic helix-loop-helix Per-ARNT-Sim

BMAL1 - brain and muscle, ARNT-like

BPDE - 7,8-diol-9,10-epoxide

bpm - beats per minute

cGMP - cyclic guanosine monophosphate

CLOCK - circadian locomotor output cycle kaput

CO - carbon monoxide

CRP - C-reactive protein

Cry - cryptochrome

CVD -cardiovascular disease

CYP - cytochrome P450

°C - degrees Celsius

DNA - deoxyribonucleic acid

eNOS - endothelial nitric oxide synthase

EROD - ethoxyresorufin-o-deethylase

ETS - environmental tobacco smoke

ET-1 - endothelin-1

FICZ - 6-formylindolo[3,2-b]carbazole

fmol - femtomoles

GST - glutathione S-transferase

GTP - guanosine triphosphate

HR - heart rate

hsp90 - heat-shock protein

IL - interleukin

IMT - intimal-media thickness ratio

ISH - isolated systolic hypertension

LD - luminal diameter

L-NAME - N(G)-nitro-L-arginine methyl ester

m - medial thickness

MD - medial diameter

mg - milligram

ml - millilitre

min - minutes

μg - micrograms

μm - micrometer

μM - micromolar

MT - medial thickness

NE - norepinephrine

NF-κB - nuclear factor-kappa B

ng - nanogram

NOx - nitrate/nitrite

NO - nitric oxide

NPAS2 - neuronal PAS protein 2

NPC1 - Niemann–Pick type C1 protein

NQO - NAD(P)H:quinone oxidoreductase

OD - outer diameter

PAHs - polycyclic aromatic hydrocarbons

PAS - Period-Arnt-Single-minded

PCBs - polychlorinated biphenyls

Per - period

pg - picogram

ppm - parts per million

ROS - reactive oxygen species

SCN - suprachiasmatic nucleus

sec - seconds

SEM - standard error of the mean

SIM - single-minded

sGC - soluble guanylyl cyclase

SMC - smooth muscle cell

TCDD - 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TNF-α - tumor necrosis factor-alpha

UGT - UDP-glucuronosyltransferase

XRE - xenobiotics response element

1.0 INTRODUCTION

1.1 Main Theme of Thesis

The major theme of this thesis is an exploration of the specific components of cigarette smoke and the pathophysiological mechanisms responsible for the smoking-induced vascular damage. In the first study of this thesis, it was confirmed that pulse wave dP/dt collected from radiotelemetry-implanted rats can be used as an indicator of active changes in arterial stiffness. In addition, the role of endothelium-derived nitric oxide in regulating dP/dt as well as the acute effects of environmental tobacco smoke (ETS) on blood pressure and arterial stiffness were examined. The remaining 2 experiments compared the effects of subchronic ETS and benzo[a]pyrene (BaP) exposure on indicators of endothelial function, inflammation, oxidative stress, arterial stiffness, circadian blood pressure patterns, and atherosclerosis.

1.2 Cardiovascular Disease and Modifiable Risk Factors

Cardiovascular disease (CVD) continues to be the leading cause of death in industrialized nations (Glynn and Rosner, 2005). The term CVD refers to a number of diseases that affect the heart and blood vessels, such as atherosclerosis, coronary heart disease, and stroke. In Canada, CVD claims the lives of over 72,000 people annually and costs the health care system over $22.2 billion in direct and indirect costs (Heart and Stroke

Foundation of Canada, cited as Anonymous, 1997). The most serious risk factors for CVD are largely modifiable, including smoking, physical inactivity, high blood cholesterol, high-fat diet, high-sodium diet, and high blood pressure. Diabetes and obesity are also major CVD risk factors that are potentially modifiable. Thus, many of the deaths and disabilities due to CVD could be prevented.

1.2.1 Cigarette Smoking

Cigarette smoking is the number one preventable risk factor for developing all major forms of cardiovascular disease, including coronary heart disease, peripheral artery disease, aortic aneurysm, stroke, and sudden death (Jonas et al., 1992; Glynn and Rosner, 2005). Even smoking as few as 1-4 cigarettes per day is associated with a higher risk of dying from cardiovascular disease (Bjartveit and Tverdal, 2005). Environmental tobacco smoke, or "secondhand smoke", has been defined as the smoke which nonsmokers are exposed to when they are in an indoor environment with smokers (McNabola et al., 2006). It is well established that chronic exposure to ETS is associated with CVD (Steenland et al., 1996, He et al., 1999; Law et al., 1997; Barnoya and Glantz, 2005; Stranges et al., 2007) and the risk for acute myocardial infarctions (AMI) in nonsmokers is markedly increased in countries where smoking prevalence is high (Pitsavos et al., 2002; Whincup et al., 2004; Teo et al., 2006). In response to this environmental health issue, several countries have instituted bans on smoking in public places and in the workplace (Meyers and Neuberger, 2008, 2009; McNabola and Gill, 2009). The effectiveness of these smoking ban policies has been widely investigated in terms of ETS concentrations, human health, and smoking prevalence (reviewed in Meyers and Neuberger, 2008, 2009; McNabola and Gill, 2009). Combined,

these studies offer consistent evidence that smoking bans have been effective in reducing smoking prevalence and ETS exposure which, in turn, is associated with a reduction in the risk for AMI in the general public, particularly in nonsmokers (Meyers and Neuberger, 2008, 2009; McNabola et al., 2009). However, ETS exposure continues to be a problem in homes and non-public spaces where current legislation does not exert any control.

1.2.2 Hypertension

Hypertension is estimated to be the leading risk factor for mortality in the world (Ezzati et al., 2002). One-quarter of the adult population in Canada and that of the entire world is hypertensive (Wolf-Maier et al., 2003). In addition, it is estimated that greater than 90% of the Canadian population will develop hypertension if they live the average life span (Chobanian et al., 2003). Because the chronic elevation in blood pressure occurs almost asymptomatically, it is a silent disorder with considerable consequences, causing cardiovascular disease and complications, including stroke, myocardial infarction, end-organ damage, and death (Hansson et al., 1998; Nichols, 2005). In fact, 42% of hypertensive individuals are unaware that their blood pressure is elevated and many of those who are aware are either undertreated or untreated (Joffres et al., 1997; Wolf-Maier et al., 2003).

Efforts to reduce the morbidity and mortality of hypertension through enhanced diagnosis, treatment, and control would require extensive resources and result in significant costs. The expenses associated with prevention and treatment of hypertension and other cardiovascular diseases can be reduced by favourably modifying risk factors. The identification and treatment of subgroups within the general population that are at highest risk of cardiovascular disease could also dramatically reduce the costs to society (Kingwell

and Gatzka, 2002; Hamilton et al., 2007). Essential hypertension is defined as a systolic blood pressure consistently over 140 mmHg or diastolic blood pressure consistently over 90 mmHg (Carretero and Oparil, 2000). This definition arose in 1939 as a result of early studies reporting that blood pressure greater than 140/90 mmHg was associated with a sharp increase in mortality (Pimenta and Oparil, 2010). In addition, blood pressure >120/80 mmHg, especially when occurring in younger individuals, was associated with progression to definitive hypertension and cardiovascular disease later in life (Pimenta and Oparil, 2010). This 'prehypertension' classification was later used by the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure to identify individuals in whom the adoption of healthy lifestyles could prevent the progressive rise in blood pressure and CVD (Chobanian et al., 2003). For example, a recent study reported that reducing dietary sodium could decrease hypertension by 30% and save an estimated $430 million per year in health care costs in Canada (Joffres et al., 2007).

Since the development and progression of cardiovascular disease is a multifactorial process, individual risk factors viewed in isolation are poor predictors of risk (Kannel, 1996; van den Hoogen et al., 2000; McVeigh et al., 2002). Thus, much research has also focused on developing methods to detect and monitor subclinical cardiovascular damage, representing the cumulative influence of multiple risk factors, in order to better assess cardiovascular risk and enable early intervention to prevent or attenuate disease progression (McVeigh et al., 2002).

1.2.3 Arterial Stiffness

Many of the traditional risk factors for cardiovascular disease, including smoking, adversely alter endothelial function as well as the structure and mechanical properties of the arterial wall (McVeigh et al., 2002). Flow-mediated vasodilation (an ultrasound-based method) has long been the accepted standard for measuring endothelial vasodilator function in humans (Nair et al., 2005). However, technical limitations and biological variability of this costly technology may reduce its research and clinical utility (Nair et al., 2005). Thus, other methods for assessing vascular function continue to be developed and tested. Deterioration in the elastic properties of the arterial wall leads to arterial stiffness, which is defined by a reduction in arterial compliance and distensibility (O'Rourke et al., 2002). Arterial stiffness has emerged as a potential alternative to flow-mediated dilation (Cohn, 2001; Nair et al., 2005) and is increasingly being recognized as a powerful cardiovascular risk factor and independent predictor of cardiovascular events (Blacher et al., 1999a, 1999b, 2000; Laurent et al., 2001).

The cushioning capacity of large arteries is essential in buffering the pulsatile pressure and flow from the heart, thereby converting intermittent blood flow to steady laminar flow. When blood is ejected from the heart during systole, the aorta expands to accommodate the stroke volume and recoils during diastole, thereby promoting forward flow. Ventricular ejection generates an incident pressure wave that is moved away from the heart at a given velocity to other arteries throughout the body. This forward wave is reflected at all points of structural and/or functional discontinuity in the arterial tree, generating a reflected pressure wave that travels backward. Thus, at any point in the vasculature, the arterial pressure wave is a resultant of incident and reflected waves. It is generally accepted that

arterial stiffness increases with advancing age and in the presence of other cardiovascular risk factors, including hypertension, hypercholesterolemia, and diabetes mellitus. In a young and healthy individual, the arteries are distensible and the pulse wave velocity is slow, therefore the reflected wave returns late to the heart, during the diastolic phase. With aging and disease processes, the arterial wall is stiffened and wave velocity is fast, causing the reflected wave to merge with the systolic part of the incident wave rather during the diastolic phase. As a result, systolic pressure and the amplitude of the wave are increased, while diastolic pressure may be decreased. This change in pulse wave velocity and altered timing of reflected waves during the cardiac cycle as a result of arterial stiffening is considered the major cause the isolated systolic hypertension (ISH) and increased pulse pressure associated with the elderly (London and Guerin, 1999; O'Rourke et al., 2002; Blacher and Safar, 2005; Nichols, 2005). Although ISH and increased pulse pressure were initially thought to be a benign consequence of aging, they are actually pathological and associated with significantly increased cardiovascular risk (Franklin et al., 2001; Wallace et al., 2007; Susic and Frohlich, 2008). In fact, a key message of the 2003 Joint National Committee report was that systolic blood pressure greater than 140 mmHg in individuals over the age of 50 is a more important CVD risk factor than diastolic blood pressure (Chobanian et al., 2003)

Structural components of the arterial wall, mainly collagen and elastin fibers, are key determinants of arterial stiffness, particularly large artery stiffness. Structural changes in the medial layer of the elastic arteries leading to increased stiffness are largely the result of progressive elastic fiber degeneration (Gkaliagkousi and Douma, 2009). An increase in stiffness related to arterial wall composition occurs with aging and is accelerated with hypertension (Gkaliagkousi and Douma, 2009). However, smooth muscle tone also

influences the stiffness of the large elastic and medium-sized muscular arteries, thereby providing functional regulation of arterial stiffness (Avolio et al., 1983, Avolio et al., 1998; Guerin et al., 2001). There are several interacting homeostatic regulators of smooth muscle tone which may be involved in the functional regulation of arterial stiffness, including the renin-angiotensin system, the autonomic nervous system, and endothelium-derived vasorelaxing and constricting factors (Zieman et al., 2005). Several studies have demonstrated the role of endothelin-1 (ET-1; McEniery et al., 2003) and nitric oxide (NO; Fitch et al., 2001; Kinlay et al., 2001; Wilkinson et al., 2002a, 2002b) in the regulation of arterial stiffness. Changes in arterial stiffness as a result of altered smooth muscle tone or structural modifications are considered an intrinsic or active change in arterial elastic properties (Stefanadis et al., 1998). However, arterial stiffness is also acutely influenced by transmural distending pressure (Nye, 1964; Cox, 1975; Stefanadis et al., 1998) and/or heart rate (Bergel, 1961; Mangoni et al., 1996), but these changes are considered to be passive (Stefanadis et al., 1998).

Recent evidence suggests that endothelial function may contribute significantly to increased arterial stiffness in aging individuals and patients with isolated systolic hypertension (Wallace et al., 2007; Ngo et al., 2009). Thus, functional changes in the arterial wall leading to increased arterial stiffness may precede clinically apparent diseases such as hypertension. Because of the high prevalence of systolic hypertension in the aging population, serious attention has been directed at precise measurement of arterial stiffness as a risk factor for hypertension and other cardiovascular diseases (O'Rourke et al., 2002; Blacher and Safar, 2005).

Various indices of arterial stiffness have been investigated in a clinical setting. Pulse pressure is often considered a surrogate measure of arterial stiffness since it reflects the pulsatile component of blood pressure and hence the buffering function of the large arteries. A number of studies indicate that pulse pressure itself has predictive value for cardiovascular events and is a predictor of CVD in various groups, including healthy individuals, hypertensive individuals, and diabetics (Hamilton et al., 2007). However, pulse pressure varies throughout the arterial tree and may only be meaningful if measured centrally (Wilkinson et al., 2001; Laurent et al., 2006; Hamilton et al., 2007; Wallace et al., 2007). Pulse wave velocity is considered the gold standard since it is a direct measure of arterial stiffness and it is often assessed in humans (O'Rourke et al., 2001, 2002). While preclinical studies have used various *in vivo* techniques to assess cardiovascular function in animals, few studies have measured arterial stiffness in rodents after ETS exposure (Liu and Fung, 1993; Guo et al., 2006), none of which were performed *in vivo*. The shape of the arterial pressure wave provides information on elastic properties of arteries and pulse wave analysis can potentially be used to quantify arterial stiffness. Blood pressure telemetry provides a large amount of reliable, sensitive physiologic data from animals and the pulse wave dP/dt can be easily extracted from arterial pressure waves. As the amplitude of the incident wave increases with arterial stiffening, the slope of rise in pressure should also increase (see Figure 1.1). Thus, we hypothesize that pulse wave dP/dt will increase with arterial stiffening and may therefore be used as an *in vivo* measure of arterial stiffness in experimental animals.

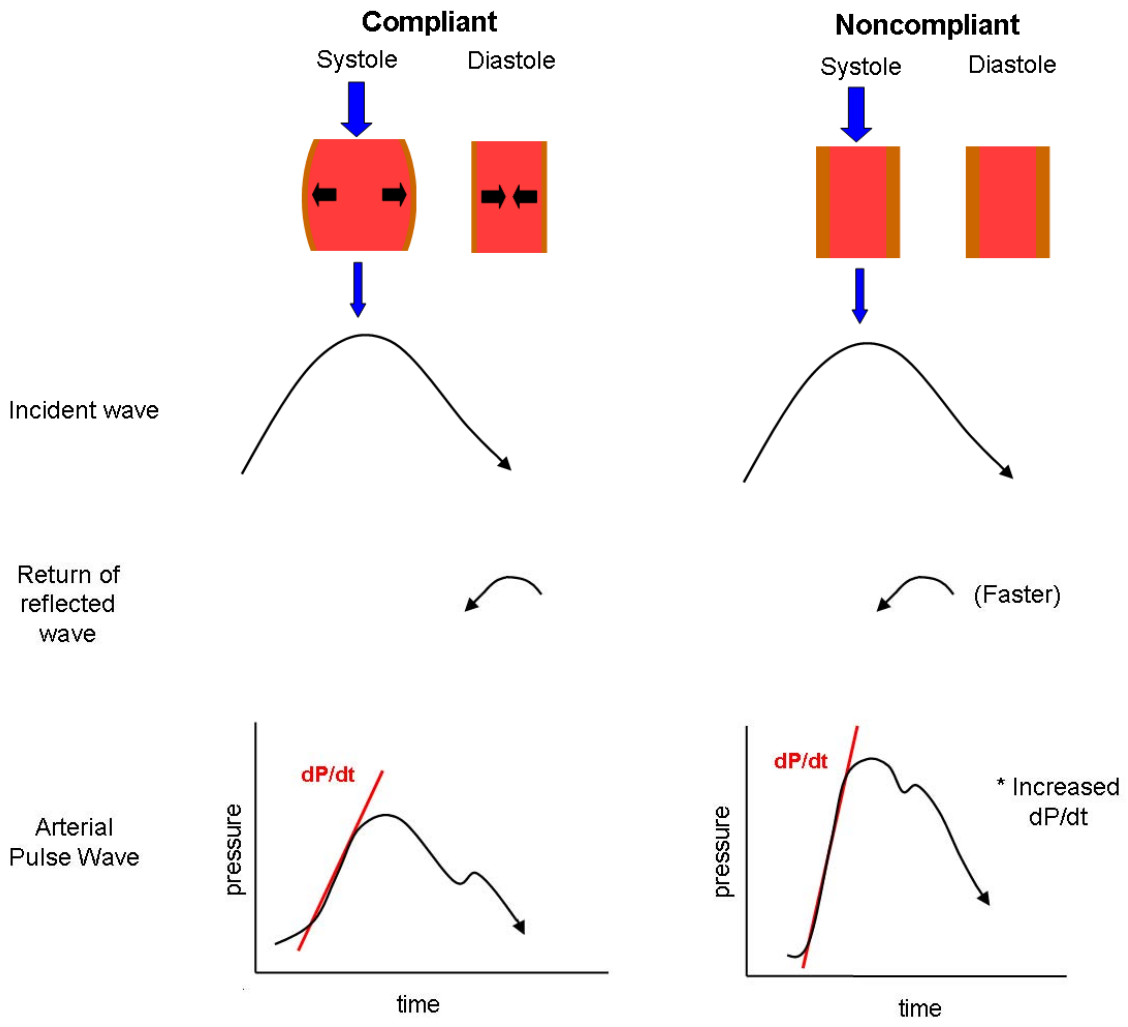


Figure 1.1 Alterations in the arterial pulse wave with arterial stiffening. When the arterial tree is compliant, arteries expand to accommodate increased blood flow during systole. The pulse wave velocity is slow and therefore the reflected wave adds to the diastolic phase. Structural and/or functional alterations of the arterial wall can lead to arterial stiffening and a reduction in arterial compliance. As a result, wave velocity throughout the arterial tree is fast and the reflected wave merges with the systolic part of the incident wave. The amplitude of the incident wave increases, as does the slope of the rise in blood pressure over time (dP/dt) (Figure modified from London and Guerin, 1999).

1.3 The Circadian Clock

Almost all living organisms exhibit a circadian rhythm, a daily cycle of biological activity based on 24-hour intervals, influenced by regular variations in the environment, such as the alternation of day/night or light/dark cycles of the sun (Reiter, 1993). Circadian rhythm is observed in most, if not all, physiological functions in mammals, including behavior, cell growth, metabolism, and immune responses (Merrow et al., 2005; Hastings et al., 2008; Schibler, 2009). The circadian "clock", the molecular basis of circadian rhythm, is found within the cells of central and peripheral tissue and provides maintenance of 24-hour periodicity in anticipation of daily changes in environmental/external conditions (Hastings et al., 2008; Schibler, 2009). Thus, circadian rhythms are regulated by 3 components: (1) the circadian "clock", (2) an "input" mechanism which allows the clock to be influenced by environmental stimuli, and (3) an "output" mechanism, which regulates physiological and behavioral processes (Dominguez-Rodriguez et al., 2009).

The molecular clock is comprised of a unique set of genes and proteins that are regulated through transcriptional, translational, and post-translational mechanisms (Ko and Takahashi, 2006). The master clock or pacemaker of circadian rhythm resides in the suprachiasmatic nucleus (SCN) of the hypothalamus, a tiny structure comprising approximately 70,000 neurons (Reppert and Weaver, 2001). The same molecular components that comprise the central clock in the SCN are also found in the peripheral clocks, including blood vessels, skeletal muscle, heart, kidney, and liver (Reppert and Weaver, 2001, 2002; Young, 2006). The central clock in the SCN processes external signals such as ambient light as well as other inputs from the brain and in turn, synchronizes the

phases of multiple peripheral clocks located in different tissues (Reppert and Weaver, 2001, 2002). Neuronal cells in the SCN maintain a near 24-hour firing pattern and regulate a variety of physiological and behavioral daily rhythms, including body temperature, sleep/awake cycles, locomotor activity, and secretion of hormones such as cortisol, melatonin, thyroxin, and vasopressin (Reiter, 1993; Reppert and Weaver, 2001, 2002). Surgical lesion of the SCN in rodents causes disruption of the behavioral circadian rhythm, which can be restored with SCN transplantation (LeSauter et al., 1996; Matsumoto et al., 1996). However, even in the absence of a master signal, the peripheral clocks still function in these rodents, suggesting that peripheral tissues possess autonomous molecular clock machinery and that regulation of the peripheral system can be uncoupled from the central clock (Yoo et al., 2004; Shimba and Watabe, 2009).

1.3.1 Circadian Rhythm and the Cardiovascular System

The cardiovascular system is known to exhibit a circadian rhythm, coordinating tissue perfusion with fluctuating functional and metabolic demands (Millar-Craig et al., 1978; Dominguez-Rodriguez et al., 2009). In humans, heart rate, cardiac output, and blood pressure all fluctuate in a diurnal (daily) manner, meaning they are generally higher during the day than at night (Dominguez-Rodriguez et al., 2009). The onset of acute cardiovascular events such as myocardial infarction, stroke, and sudden cardiac dealth also exhibit circadian variations, with increased frequencies in the period from 6:00 AM to noon (Muller et al., 1987, 1989; Elliot, 1998). For many years, rhythms in the cardiovascular system were primarily attributed to diurnal variations in autonomic nervous system activity (driven by the SCN) (Dominguez-Rodriguez et al., 2009). However, the ability of the cardiovascular system

to respond to neural and humoral stimuli in an appropriate and timely manner appears to be equally important. Circadian clocks have been identified in every mammalian cell investigated to date, including vascular smooth muscle cells (SMCs) and cardiomyocytes (Davidson et al., 2005; Durgan et al., 2005; Edery, 2000; McNamara et al., 2001). Intracellular circadian clocks provide a mechanism by which the heart and vasculature can influence cardiovascular responses (Dominguez-Rodriguez et al., 2009). It is believed that the clocks allow a cell to "anticipate" diurnal variations in stimuli, such as autonomic nervous activity, thereby synchronizing the response of the cell with the timing of the stimulus (Dunlap, 1999; Edery, 2000; Yamazaki et al., 2000; Whitmore et al., 2000). Thus, the variations in cardiovascular function throughout the day are the result of a complex interplay between environmental stimuli and intrinsic mechanisms (i.e., central and peripheral circadian clocks) (Dominguez-Rodriguez et al., 2009).

There is increasing evidence that attenuation or malfunction of the circadian clock or a loss of synchronization between central and peripheral clocks may contribute to the development of cardiovascular disease (Dominguez-Rodriguez et al., 2009). Such a loss of synchronization occurs in shift workers, who have a 40% increase in risk for developing cardiovascular disease (Sundberg et al., 1988; Boggild and Knutsson, 1999; Chau et al., 1989). There is also evidence that circadian clocks are altered in various animal models of CVD. For example, the rhythmic expression of genes regulated by the circadian clock has been reported to be attenuated in the heart of rats during left ventricular hypertrophy (Young et al., 2001). Another study reported that in a rat model of hypertension, the circadian variation in blood pressure was altered as well as the rhythmic expression of clock genes and clock-regulated genes in the hypertrophic heart (Mohri et al., 2003). Studies using rodent

models in which clock genes are altered through genetic or environmental means also implicate disruption of the circadian clock as a significant factor in the pathogenesis of cardiovascular diseases. For example, a recent study reported endothelial dysfunction, vascular injury and increased pathological remodeling in mice with mutated clock genes (Anea et al., 2009). Thus, alterations in circadian rhythm may play a role in both acute cardiovascular events and chronic progression of disease.

1.3.2 The Circadian Clock and Hypertension

It has long been known that circadian rhythms are a characteristic feature of blood pressure regulation and hypertension (Rudic and Fulton, 2009). In the absence of disease, blood pressure values are higher while awake with mental and physical activity, but decrease during rest and sleep, only to increase again in early morning during the transition of sleep to wakefulness (Kario et al., 2003). This pattern arises not only from exogenous patterns of activity, stress, and posture but also from endogenous circadian rhythms (Rudic and Fulton, 2009). In humans, a nocturnal fall or dip in blood pressure of about 10-20% is considered normal, and such individuals are termed dippers (Pickering and Kario, 2001). In hypertensive patients, the rhythm and pattern of blood pressure oscillations can frequently be abnormal, with patients presenting as extreme dippers (>20% decrease in nocturnal blood pressure), non-dippers (<10% decrease in nocturnal blood pressure) and reverse-dippers (increased nocturnal blood pressure) (Kario et al., 1996, 2003; Pickering and Kario, 2001). Hypertensive subjects with non-dipping patterns are at increased risk of target-organ damage, cardiovascular events, and death (Verdecchia et al., 1994; Pickering and Kario, 2001;

Ingelsson et al., 2006; Fagard et al., 2009). However, the mechanisms underlying the loss of nocturnal blood pressure dipping are not completely understood.

The circadian clock influences multiple mechanisms of blood pressure regulation (reviewed in Rudic and Fulton, 2009). As the master clock to all peripheral rhythms, the SCN plays a role in blood pressure rhythm via neural and humoral signals (Sano et al., 1995; Dominguez-Rodriguez et al., 2009). For example, locomotor activity follows a circadian rhythm that originates in the SCN (Sano et al., 1995). Day to night differences in mental and physical activity (behavior) appear to be major determinants of blood pressure rhythm (Degaute et al., 1991; Dominguez-Rodriguez et al., 2009). Behavioral aberrations (e.g. shift work, sleep apnea, and inadequate sleep) can impair central clock function and significantly affect the circadian variation in blood pressure (Rudic and Fulton, 2009). Diurnal variation in the autonomic nervous system activity is a main suspect in mediating blood pressure rhythms. In support of this, autonomic failure patients have been reported to exhibit non-dipping blood pressure (Mann et al., 1983; Omboni et al., 2001). The melatoninergic system may also be important. The master clock of the SCN stimulates the pineal gland to produce melatonin at night in response to the phase-shifting actions of lights. As a result, normal plasma melatonin concentrations are low during the day and increase at night (Dominguez-Rodriguez et al., 2009). Melatonin receptors are expressed in both the brain and the cardiovascular system (Viswanathan et al., 1990; Dubocovich and Markowska, 2005; Ekmekcioglu et al., 2001). In hypertensive patients with impaired circadian blood pressure rhythms, melatonin supplementation has been reported to improve the nocturnal blood pressure dipping (Scheer et al., 2004).

The rhythmic oscillations of blood pressure are not only the consequence of activity/locomotor/behavioural rhythms, but also guided by circadian actions in the periphery (reviewed in Rudic and Fulton, 2009). Endothelial function (Keskil et al., 1996) and vasomotor tone exhibit a circadian rhythm (Panza et al., 1991) and nitric oxide has been implicated in the control of blood pressure rhythm (Witte et al., 1995; Rudic and Fulton, 2009). In addition, increased production of reactive oxygen species (ROS) can disrupt the timing of circadian clock (Hardeland et al., 2003). Thus, multiple mechanisms could account for a reduction in nocturnal blood pressure dipping.

1.3.3 The Molecular Clock and the Aryl Hydrocarbon Receptor

The core signaling pathway of the molecular clock is comprised of a positive limb of transcription factors (brain and muscle, ARNT-like 1 or BMAL1; circadian locomotor output cycle kaput or CLOCK; neuronal Per-ARNT-Sim protein 2 or NPAS2) and a negative limb of regulatory proteins (Period or PER1, PER2, PER3, and Cryptochrome or CRY1, CRY2). BMAL1 forms a heterodimer with either CLOCK or NPAS2 and drives transcription from E-box elements found in the promoter of circadian-responsive genes, including hormones and enzymes as well as *Per* and *Cry*. After translation, the PER and CRY proteins form various complexes and inhibit the transcription driven by BMAL1 and CLOCK, including their own, thereby completing the negative feedback loop (Dunlap, 1999; Young and Kay, 2001; Reppert and Weaver, 2002).

Many of the proteins of the molecular clock belong to the basic helix-loop-helix Period-ARNT-Single-minded (bHLH/PAS) gene family, including CLOCK, BMAL1, and NPAS2 (Ko and Takahashi, 2006). This family of transcriptional regulatory proteins is

characterized by a PAS domain, composed of 50 amino acid repeats, and a basic helix-loop-helix domain. The term "PAS" is derived from the first 3 members of the family: the period gene, the aryl hydrocarbon receptor nuclear translocator (ARNT) gene, and the single-minded (SIM) gene (Mukai et al., 2008; Shimba and Watabe, 2009). The PAS domain is found in various proteins that play a role in development and adaptation to the environment, affecting circadian rhythms (Sassone-Corsi, 1997), hypoxia (Semenza, 1998), and xenobiotics metabolism (Hankinson, 1995).

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor whose physiological role is largely unclear. It has been extensively studied for its role in mediating the toxicity of environmental contaminants, such as halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). Activation of the AhR by agonists such as PAHs leads to nuclear translocation of the agonist-bound AhR, followed by its dimerization with the ARNT (Figure 1.2) (Nebert et al., 2000). The AhR:ARNT heterodimer binds to the xenobiotic response element (XRE), also known as the dioxin response element, to induce transcription of a battery of AhR-responsive genes. Among these genes are the xenobiotic-metabolizing enzymes such as cytochrome P4501A1 (CYP1A1) (Nebert et al., 2000).

Evolutionary conservation of the AhR and the abnormal phenotype of AhR null mice suggest a role for the AhR in physiological functions. However, endogenous ligands of the AhR remain elusive and its physiological role remains unknown. Both the AhR and the ARNT are bHLH/PAS domain proteins (Hahn, 1998). The ARNT amino acid sequence shows high homology to the BMAL1 protein (Hogenesch et al., 1998; Takahata et al., 1998), which is known as a master regulator of circadian rhythm (Bunger et al., 2000). Because of this structural similarity, BMAL1 is also called ARNT3 (Takahata et al., 1998). The

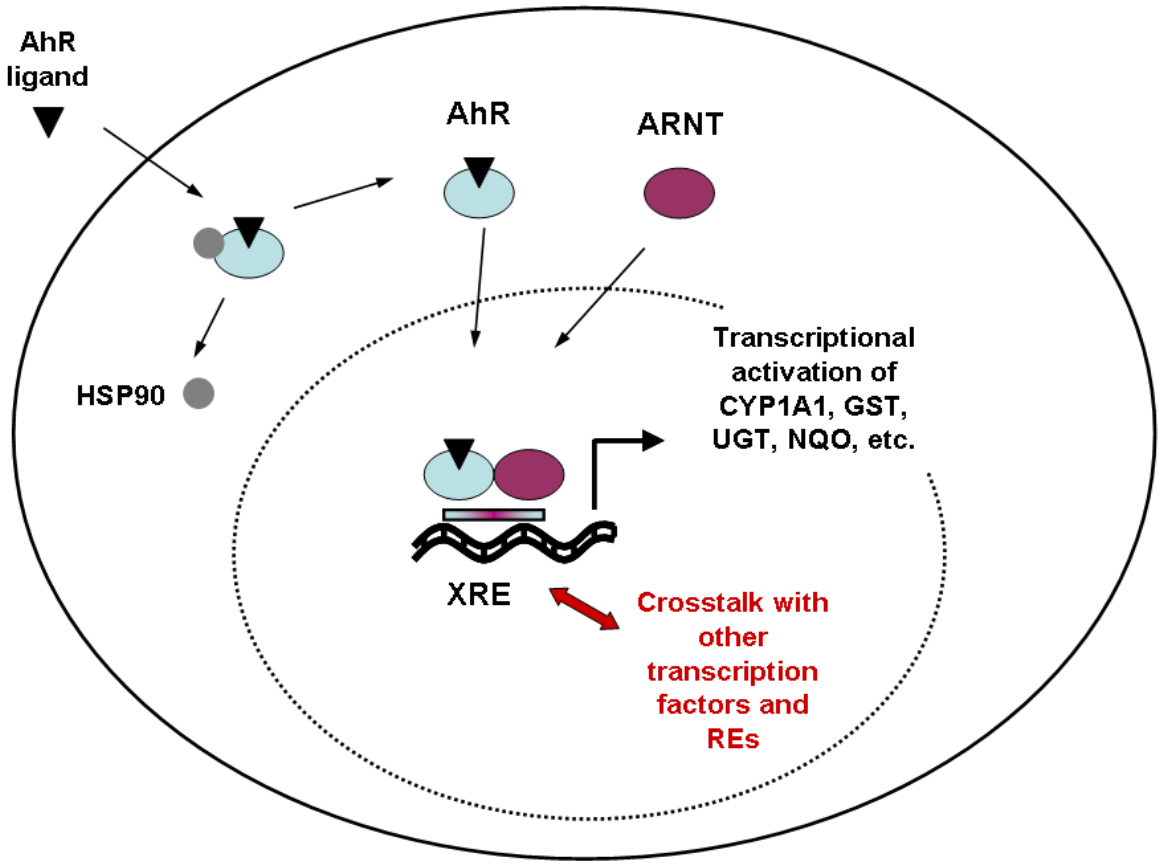


Figure 1.2 Activation of the aryl hydrocarbon receptor (AhR). The AhR exists in the cytosol as a complex with 2 molecules of a 90 kDa heat-shock protein (hsp90) and other proteins. Binding of the ligand results in the dissociation of the hsp90 and the translocation of the AhR to the nucleus, where it binds the AhR nuclear translocator (ARNT). The AhR-ARNT heterodimer then activates transcription by binding to xenobiotic-responsive elements (XRE), located in the promoter region upstream of the target genes such as cytochrome P450 1A1 (CYP1A1) (Hahn, 1998). RE - response element, UGT - UDP-glucuronosyltransferase, NQO - NAD(P)H:quinone oxidoreductase, GST - glutathione S-transferase.

similarity of the AhR and ARNT with the circadian clock genes suggests the potential involvement of the AhR in regulation of circadian rhythms. Photoproducts of tryptophan, including 6-formylindolo[3,2-b]carbazole (FICZ), may act as transducers of light and have a high affinity for the AhR (Rannug et al., 1987, 1995). Thus, tryptophan products have been suggested as potential endogenous AhR ligands (Rannug and Fritsche, 2006). In support of this, a recent study reported that FICZ blocked glutamate-induced phase shifting in SCN cells (Mukai and Tischkau, 2007). Previous studies also indicate that there are daily rhythms of AhR and ARNT protein expression in various tissues, including the liver and lung (Richardson et al., 1998; Shimba and Watabe, 2009). Diurnal expression of AhR mRNA as well as its downstream target, CYP1A1, has also been reported in tissues such as the liver and the SCN (Huang et al., 2002; Mukai et al., 2008; Shimba and Watabe, 2009). Thus, similar to many other circadian clock genes, AhR-related genes exhibit a circadian rhythm.

A recent study reported that behavioral rhythmicity is normal in AhR-knock out mice (Mukai et al., 2008). However, while deletion of 2 circadian clock genes often results in clear behavioral arrhythmicity, deletion of a single circadian clock gene rarely causes complete disruption of rhythmicity (van der Horst et al., 1999; Zheng et al., 2001; Dudley et al., 2003; Mukai et al., 2008). Thus, the AhR may share redundant roles with other PAS domain proteins and its involvement in circadian rhythms cannot be ruled out (Mukai et al., 2008). While the AhR may not play a role in behavioural activity rhythm, it may have evolved to play different roles in circadian timing among different tissues and organs. For example, the same study also reported that exposure to the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreased phase shifts in response to light as well as altering liver and SCN expression of PER1 and BMAL1 in mice (Mukai et al., 2008). Thus, chronic activation of the

AhR may affect the ability of the circadian timekeeping system to adjust to alterations in environmental lighting by altering circadian clock genes (Mukai et al., 2008). In addition to behavioural rhythms, TCDD exposure has been reported to disrupt circadian rhythms in feeding behaviour (Seefeld et al., 1984; Kelling et al., 1985), hormones (Jones et al., 1987; Pohjanvirta et al., 1989; Yellon et al., 2000) and immune systems (Garrett and Gasiewicz, 2006).

Ambulatory blood pressure monitoring is increasingly being used in humans to evaluate the effects of lifestyle (e.g. smoking) on blood pressure. It provides greater statistical power than casual measurements and allows for the analysis of circadian rhythms in blood pressure (Pickering, 1990; Pickering et al., 1995; Beilin, 2002). In nocturnal animals such as rats, heart rate, blood pressure, and cardiac output also exhibit a circadian rhythm. These parameters are greatest during the dark phase when the animal is most active and searches for food, and decrease during the light phase when the animal is less active or sleeping (van den Buuse, 1999). Exposure to TCDD has been reported to cause hypertension in mice with a loss in the dipping pattern of diurnal blood pressure (Kopf et al., 2008). In contrast, AhR null mice are also hypertensive, although the circadian rhythm of blood pressure was not determined (Lund et al., 2003). Polycyclic aromatic hydrocarbons are ubiquitous environmental components that arise from multiple sources, including industrial processes, vehicle exhaust, or cooking of foods (Miller and Ramos, 2001). Polycyclic aromatic hydrocarbons, inclucing BaP, and other AhR ligands are also found in cigarette smoke (Hoffmann and Hoffmann, 1997). A previous study reported that stress and cigarette smoke exposure in rats altered the daily rhythm characteristics of heart rate, body temperature, and locomotor activity (Pelissier et al., 1997), but effects on blood pressure are

not clear. Thus, a major goal of this study was to examine alterations in the circadian rhythm of blood pressure after exposure to ETS and BaP.

1.4 Mechanisms of Cardiovascular Disease

Although epidemiologic evidence has clearly established an association between exposure to cigarette smoke and CVD, the exact components of cigarette smoke responsible and their pathophysiological mechanisms have not been clearly elucidated. One possible unifying hypothesis for the effects of CVD risk factors, including cigarette smoking and hypertension, is that they increase production of ROS, which would initiate several processes involved in atherogenesis, such as endothelial dysfunction and inflammation (Raupach et al., 2006). However, the exact time course of each pathophysiological change in function during the early stages of CVD requires further investigation.

1.4.1 Atherogenesis

Atherosclerosis is a complex process involving a variety of structural and functional changes in the arterial wall. It involves the interaction of several cell types with the endothelium, including monocytes, platelets, and smooth muscle cells. The end result is the formation of fibrous atherosclerotic plaques in the tunica intima and media of large- and medium-sized arteries, particularly those located in areas of high blood pressure and turbulence (Wang and Wang, 2005). Endothelial dysfunction, inflammation, and modification of lipids are all key early events in the initiation and progression of

atherosclerosis (Ross, 1993). During the early stages of plaque formation, oxidized low density lipoproteins (LDLs) are taken up by macrophages in the subendothelial space, leading to the formation of foam cells and "fatty streaks" (Ross, 1993). Abnormal proliferation and migration of vascular smooth muscle cells from the medial layer into the intimal space of the artery are also key early events in atherosclerosis (Ross, 1993; Nakashima et al., 2007).

There are various hypotheses for the initiating event in atherogenesis. The "oxidative modification hypothesis" is currently the major hypothesis for the initiating event in atherosclerosis (Stocker and Keaney, 2005). This hypothesis suggests that increased oxidative stress and generation of ROS in the vascular wall leads to oxidation of LDL, which then mediates a multitude of effects on the vascular wall. For example, oxidized LDL has been shown to attract circulating monocytes into the intimal space and induce adhesion to the endothelium, promote foam cell formation, injure endothelial and other cells of the vascular wall (via both apoptosis and necrosis), interfere with endothelium-dependent vasodilation, and modulate mitogenic signaling in vascular SMCs leading to migration and proliferation (Stocker and Keaney, 2005). In contrast, the monoclonal hypothesis is based on the molecular links that exist between atherosclerosis and carcinogenesis. Both diseases are characterized by uncontrolled cellular proliferation and dedifferentiation which may arise through the mutation of proto-oncogenes. The monoclonal hypothesis states that the formation of atherosclerotic lesions result from proliferation of a transformed SMC phenotype and can be viewed as benign smooth muscle cell tumors of the artery wall (Ramos and Partridge, 2005). Although the oxidative modification hypothesis is currently favoured over the monoclonal hypothesis for atherogenesis, neither has been disproven. Furthermore,

these hypotheses are not mutually exclusive and potentially both mechanisms may contribute to atherosclerosis.

1.4.2 Endothelial Dysfunction

The endothelium is localized at the interface between the vessel wall and circulating blood and it regulates numerous homeostatic functions. Signaling molecules derived from the endothelium play a pivotal role in the regulation of vascular tone, platelet activation and aggregation, thrombosis, and fibrinolysis. In addition, the endothelium regulates and directs the inflammatory process through the production of cytokines and adhesion molecules (Barnoya and Glantz, 2005). Injury or activation of the endothelium (e.g. by cytokines, ROS, or circulating xenobiotics) disrupts these normal regulatory mechanisms, resulting in structural and functional alterations of the arterial wall commonly referred to as endothelial dysfunction (Rubanyi, 1993).

The endothelium contributes to the regulation of smooth muscle tone and blood pressure through the synthesis and release of vasorelaxing (nitric oxide, prostacyclin, endothelial-derived hyperpolarizing factor) and vasoconstricting factors (ET-1, arachidonic acid metabolites, angiotensin II or AngII) (Lüscher, 1990; Rubanyi, 1993). Thus, one manifestation of endothelial dysfunction is an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors leading to impaired vascular reactivity. Although it seems likely that functional abnormalities of the endothelium would precede structural changes during the atherosclerotic process, this has not been clearly established. Furthermore, although there is currently a disproportionate focus on NO in research, other factors are likely to be contributing to the pathogenic process.

In addition to functioning as mutual antagonists in the regulation of smooth muscle tone and blood pressure (Gray and Webb, 1996; Ignarro et al., 1987), NO and ET-1 play opposing roles in other processes involved in atherogenesis. While NO possesses antiatherosclerotic properties, ET-1 acts as a proatherosclerotic factor in the modulation of platelet activity, thrombosis, growth and proliferation of vascular SMCs, and leukocyte adhesion (Moncada and Higgs, 1993; Levin, 1995; De Meyer and Herman, 1997; Schulz et al., 2004). Thus, an imbalance between NO and ET-1 actions can contribute to endothelial dysfunction beyond the regulation of smooth muscle tone and plays a key role in the development of atherosclerosis and CVD (Mather et al., 2002).

1.4.3 Oxidative Stress

Classically, endothelial dysfunction has been defined as a decrease in NO bioactivity. Many of the protective endothelial functions are mediated through the NO pathway, such as maintaining SMCs in a non-proliferative state and causing relaxation of smooth muscle (Figure 1.3). In addition, NO also limits several other atherogenic processes, including inflammation, platelet activation, leukocyte adhesion, and thrombosis (Napoli and Ignarro, 2001). Thus, an alteration in the bioactivity or production of NO is a maladaptive event and is known to be one of the earliest alterations in the atherosclerotic process (Schulz et al., 2004). A loss in NO bioactivity is commonly assessed in humans and experimental animals by measuring endothelium-dependent vasodilation (Schulz et al., 2004). Impaired endothelial-dependent vasodilation is frequently encountered in the presence of cardiovascular risk factors such as smoking and hypertension (Hadi et al., 2005) and serves as an independent predictor of future cardiovascular events (Schachinger et al., 2000).

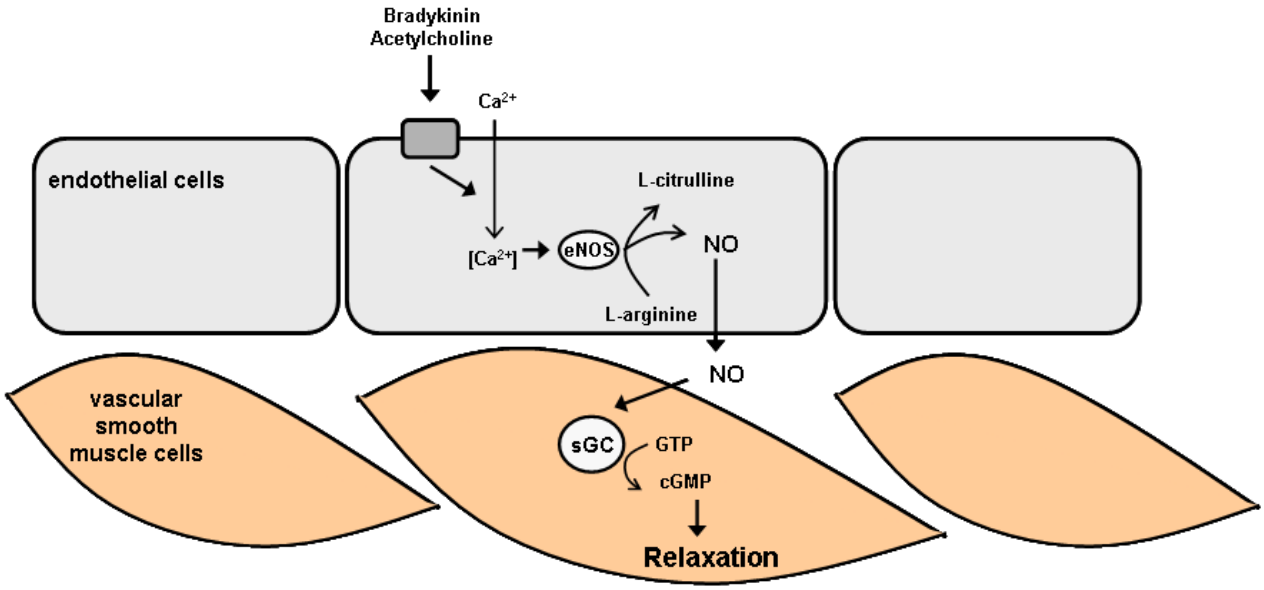


Figure 1.3 The nitric oxide-cyclic guanosine monophosphate pathway. In response to various stimuli such as acetylcholine, bradykinin, and increased shear stress, endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO) from L-arginine. The NO is released from the endothelial cell and diffuses into adjacent smooth muscle cell (SMC) and activates guanylyl cyclase, resulting in the formation of cyclic guanosine monophosphate (cGMP). cGMP, in turn, causes SMC relaxation and maintains SMC in a non-proliferative phenotype. soluble guanylyl cyclase (sGC), guanosine triphosphate (GTP). Figure modified from Pilz and Casteel, 2003; Münzel et al., 2005.

There are various potential abnormalities that could lead to impaired endothelium-dependent vasodilation, such as increased NO degradation or decreased activity and/or expression of endothelial nitric oxide synthase (eNOS) leading to decreased NO production. In most situations of endothelial dysfunction in diseased patients, the expression of eNOS has been shown to be paradoxically increased in the vascular tissue (Münzel et al., 2005). This suggests that the eNOS expression itself is not limiting NO levels, but that the enzyme may have a limited ability to produce NO, and/or that NO is being inactivated before it can mediate vasodilation. Although the mechanisms underlying endothelial dysfunction may be multifactorial, it has been shown that the production of ROS may be a key player in this phenomenon. Increased oxidative stress and the production of ROS has been shown to occur within the endothelium, smooth muscle cell layer, and the adventitia, all of which play an important role in the development of endothelial dysfunction (Münzel et al., 2005). Superoxide anion rapidly reacts with NO to form the highly reactive peroxynitrite, thereby biologically inactivating NO before it can cause SMC relaxation. At high concentrations peroxynitrite is cytotoxic and may cause oxidative damage to DNA, proteins, and lipids (Beckman, 1996; Zou and Ulrich, 1996). Peroxynitrite has a propensity to interact with tyrosine residues to form nitrotyrosine, which can disrupt protein function. Nitrotyrosine can also be used as a surrogate measure of the highly unstable peroxynitrite. Importantly, peroxynitrite has been shown to disrupt the zinc-thiolate cluster of eNOS, resulting in uncoupling of the enzyme and inhibition of NO production (Zou et al., 2002). Not only could uncoupled eNOS contribute to endothelial function through reduced NO production, but it also becomes a source of ROS itself. In its uncoupled state, eNOS transfers electrons to molecular oxygen instead of L-arginine, resulting in the production of superoxide instead of

NO and further increasing oxidative stress (Münzel et al., 2005). Thus, the net effect of oxidative stress within the vessel is to decrease available NO and impair endothelial function.

Endothelin-1 is described as the most potent vasoconstrictor known (Yanagisawa et al., 1988). It is synthesized within endothelial cells and can act in a paracrine or an autocrine manner by binding to ET type A (ETA) or ET type B (ETB) receptors on adjacent endothelial and vascular smooth muscle cells. The binding of ET-1 to ETA and ETB receptors on smooth muscle cells results in vasoconstriction, cell proliferation, inflammation, and fibrosis (Levin, 1995; Iglarz and Clozel, 2007). Numerous pathologic conditions, including hypertension and atherosclerosis, are often associated with chronic elevations of circulating ET-1 levels and/or modified ET receptor distribution (Kaplan, 2002; Clozel, 2003; Migneault et al., 2005; Iglarz and Clozel, 2007). Not only does ET-1 exert vasoconstriction, but there is also evidence that increased ET-1 can induce endothelial dysfunction by increasing oxidative stress in the vessel wall and reducing NO bioactivity (Duerrschmidt et al., 2000; Li et al., 2003; Amiri et al., 2004; Migneault et al., 2005). Clinical data also indicate that ETA and ETB receptor antagonists can improve NO bioactivity and endothelial function in pathological conditions (Clozel, 2003). Thus, beyond its vasoconstrictive properties, ET-1 may increase the susceptibility to atherosclerosis by increasing oxidative stress in the vessel wall leading to endothelial dysfunction.

1.4.4 Inflammation

Vascular wall inflammation plays a key role in the pathogenesis of atherosclerosis, cardiovascular disease, and hypertension (Ross, 1999; Brasier et al., 2002; Virdis and Schiffrin, 2003; Schiffrin and Touyz, 2004; Touyz and Schiffrin, 2004; Schiffrin, 2005;

Savoia and Schiffrin, 2006). Atherosclerosis is generally viewed as an inflammatory condition, with leukocyte recruitment and expression of pro-inflammatory cytokines occurring early in the atherogenic process (Ross, 1999; Libby, 2002). The inflammatory reaction involves complex interactions between vascular cells and inflammatory cells, such as neutrophils and macrophages. This interaction induces an inflammatory response in vascular cells through the increased expression of cytokines, chemokines, adhesion molecules, matrix metalloproteinases, and growth factors (Ross, 1999). The inflammatory component which plays an integral role in the pathogenesis of atherosclerosis (Libby, 2002) is also present in other conditions associated with cardiovascular disease, including hypertension (Chae et al., 2001; Savoia and Schiffrin, 2006) and diabetes (Festa et al., 2000). In fact, hypertension may also be considered a low-grade inflammatory disease (Savoia and Schiffrin, 2006). C-reactive protein (CRP), a marker for systemic inflammation, is associated with increased cardiovascular morbidity and mortality (Ridker et al., 2000; Hackam and Anand, 2003). Elevated CRP levels have also been previously associated with increased pulse pressure in normotensive (Abramson et al., 2002) and hypertensive individuals (Schillaci et al., 2003). Furthermore, mildly elevated CRP detected with high sensitivity assays (often referred to as hsCRP) has been shown to be associated with future development of hypertension, (Sesso et al., 2003).

Circulating and in situ-activated neutrophils and macrophages generate ROS (Rennard and Daughton, 1993) which may serve as secondary messengers in multiple signaling pathways, including inflammatory processes and cytokine production (Forman et al., 2002). Indeed, circulating leukocytes and plasma inflammatory markers and cytokines have been reported to be elevated in smokers (Winkel and Statland, 1981; Abboud et al.,

1986; McKarns et al., 1995; Tracy et al., 1997; Mazzone et al., 2001; Bermudez et al., 2002) and in patients with hypertension (Pauletto and Rattazzi, 2006) or cardiovascular disease (Lind, 2003). Proinflammatory cytokines themselves have also been shown to induce oxygen free radical production in other cells, including vascular smooth muscle cells (Meier et al., 1989; Radeke et al., 1990; De Keulenaer et al., 1998). In addition to stimulating the production of oxygen free radicals, cytokines appear to be important mediators of endothelial dysfunction and injury (Zhang, 2008). Furthermore, CRP has been reported to induce ET-1 production in cultured venous endothelial cells (Verma et al., 2002) and there is also evidence that CRP can directly alter endothelial NO production (Venugopal et al., 2002; Verma et al., 2002). Thus, it has been hypothesized that circulating inflammatory mediators could be involved in the pathological alteration of vascular tone regulation (Pauletto and Rattazzi, 2006).

In support of this, acute systemic inflammation induced by vaccination has been reported to impair endothelium-dependent vasodilation (Hingorani et al., 2000) and increase arterial stiffness (Vlachopoulos et al., 2005) in humans. Elevated CRP levels are associated with impaired endothelial vasodilatory function in patients with coronary artery disease (Fichtlscherer et al., 2000). A large number of studies have also demonstrated a positive relationship between CRP and arterial stiffness (Mattace-Raso et al., 2004, Duprez et al., 2005; Kullo et al., 2005; Pietri et al., 2006). It has been reported that arterial stiffness is independently correlated to CRP levels in patients with inflammatory diseases such as rheumatoid arthritis (Mäki-Petäjä et al., 2006) and systemic vasculitis (Booth et al., 2004). In essential hypertension, independent correlations have been shown between arterial stiffness and levels of CRP, TNF-α, and IL-6 (Mahmud and Feely, 2005; Pietri et al., 2006). A strong

correlation between CRP levels and arterial stiffness has also been reported in healthy individuals, further supporting a role of inflammation in the process of arterial stiffening even in health (Yasmin et al., 2004). Thus, there is considerable evidence that inflammation participates in the development and pathogenesis of hypertension as well as alterations in vascular function (Savoia and Schiffrin, 2006). While the precise mechanisms are not yet clear, it appears that oxidative stress and reduced NO bioactivity may play an important role.

1.5 Cigarette Smoke and Oxidative Stress

As previously discussed, oxidative stress is generally believed to play a role in endothelial dysfunction and atherogenesis. Exposure to ETS produces oxidative stress (Howard et al., 1998a) and there are a number of potential sources of free radicals upon exposure to cigarette smoke. Both the particulate and vapour phase of cigarette smoke contain high concentrations of ROS, NO, peroxynitrite, and free radicals of organic compounds (Pryor and Stone, 1993). In addition to these short-lived, highly reactive substances, the vapour phase also contains stable substances that have the potential to increase the intracellular production of ROS (Stedman, 1968). Free radicals could also arise from macrophages and neutrophils as well as other endogenous sources of ROS, including uncoupled eNOS, xanthine oxidase, and the mitochondrial electron transport chain (Pryor and Stone, 1993; Smith and Fischer, 2001; Ambrose and Barua, 2004; Rahman and Laher, 2007).

Various surrogate measures have been used to document the oxidative stress generated by cigarette smoke exposure, including a decrease in antioxidant levels and an

increase in oxidative stress biomarkers (Barnoya and Glantz, 2005). Studies have shown that antioxidants are capable of inhibiting some of the adverse effects of smoking, such as the increase in blood pressure, endothelial dysfunction, and arterial stiffness (Heitzer et al., 1996; Mays et al., 1999; Papamichael et al., 2004, 2006; Karatzi et al., 2007; Peluffo et al., 2009). Such findings support the concept that CVD risk factors such as smoking increase oxidative stress, which then initiates processes involved in atherogenesis.

1.6 Cigarette Smoke Components

Cigarette smoke contains over 4,000 known components and can be divided into a vapour phase and a particulate phase. The vapour phase contains volatile components such as carbon monoxide (CO), acrolein, acetaldehyde, and 1,3-butadiene (Hoffmann and Hoffmann, 1997). The particulate or 'tar' phase contains the semi- and non-volatile components, including nicotine and PAHs such as BaP (Hoffmann and Hoffmann, 1997). The majority of ETS is made up of smoke that emerges from the tip of the burning cigarette. This is called sidestream smoke and constitutes about 85% of ETS. The remainder is made up of mainstream smoke, which has been inhaled then exhaled by the active smoker. Several toxins have been shown to be up to 100-fold higher in sidestream smoke compared to mainstream smoke (Lofroth and Rannung, 1988).

The majority of studies have focused on the effects of cigarette smoke as a whole, with few single components being studied extensively in isolation. The constituents of cigarette smoke that have been best studied in isolation are nicotine and carbon monoxide. Although known to have acute effects, their long-term effects are less well-known.

Epidemiological and experimental studies suggest that CO does not initiate or accelerate atherosclerotic plaque formation (Smith and Steichen, 1993; Strom et al., 1995). In humans and experimental animals, acute nicotine exposure has been implicated in a number of the mechanisms involved in atherogenesis, including endothelial dysfunction, ROS production, inflammation, and reduced NO bioactivity (Rahman and Laher, 2007). However, the increased levels of oxidative stress observed in chronic smokers are not mimicked by exposure to nicotine alone (Reilly et al., 1996). In a recent study, nicotine was reported to acutely increase arterial stiffness in humans (Adamopoulos et al., 2009). Short-term nicotine administration has also been shown to alter the circadian rhythm of blood pressure in humans (Benowitz et al., 2002; Yugar-Toledo et al., 2005), with increased blood pressure in the morning and a loss of the nocturnal dipping pattern (Yugar-Toledo et al., 2005). However, the chronic effects of nicotine on arterial stiffness and circadian blood pressure patterns may yield different results since chronic nicotine exposure is associated with desensitization of nicotinic receptors (Gentry and Lukas, 2002). Overall, the majority of current evidence suggests that, at levels associated with cigarette smoking and more so with levels associated with ETS, nicotine has a minor effect on the initiation or progression of atherosclerosis in experimental animals (Smith and Fischer, 2001; Sun et al., 2001). Thus, the increase in atherosclerosis associated with cigarette smoke exposure does not appear to be attributable to CO or nicotine alone (Smith and Fischer, 2001).

Other studies using single components of cigarette smoke have shown that the volatile components, such as acrolein, acetaldehyde, and 1,3-butadiene, are cytotoxic to cells of the vessel wall (Ambalavanan et al., 2001; Penn et al., 2001). Chronic exposure to cigarette smoke has been shown to result in the acceleration of plaque development in

humans (Howard et al., 1998b) and experimental animals (Penn and Snyder, 1993; Penn et al., 1994), even in the absence of high fat and/or high cholesterol intake. This may occur through the formation of reactive metabolites and DNA adducts. PAHs such as BaP are components of the tar phase of cigarette smoke and have been implicated in the initiation and progression of atherosclerosis. PAHs are capable of generating oxidative stress and forming reactive metabolites which may lead to vascular injury via inflammatory reactions, endothelial injury, vascular SMC proliferation, DNA damage/mutations, cellular apoptosis, and initiation of foam cell formation (Nel et al., 2001; Miller and Ramos, 2001; N'Diaye, 2006; Knaapen et al., 2007; Oesterling et al., 2008; Podechard et al., 2008; Zhang and Ramos, 2008; Podechard et al., 2009; Meng et al., 2009; Wang et al., 2009; Yang et al., 2009), all of which contribute to acceleration of atherosclerosis. Therefore, this project will focus on PAHs as the possible agents responsible for mediating the adverse effects of cigarette smoke exposure in the cardiovascular system, including increased oxidative stress, inflammation, arterial stiffness, and atherosclerosis.

1.7 Cigarette Smoke and Cardiovascular Disease: Clinical and Experimental Studies

1.7.1 Cigarette Smoke and Endothelial Function/Vasorelaxation

Increased ROS as a result of smoking may lead to biological inactivation of NO before it can mediate vasorelaxation and increased formation of peroxynitrite (Beckman, 1996; Zou and Ullrich, 1996). Alternatively, cigarette smoke components may cause

endothelial dysfunction directly by interfering with the production of NO or via cytotoxic effects on endothelial cells. Formaldehyde, a gas phase component of cigarette smoke, has been reported to inhibit the bradykinin-induced increase the intracellular calcium in pig aortic endothelial cells (Mazak et al., 2002). In contrast, there is evidence that carbon monoxide may cause acute vasorelaxation indirectly through formation of carboxyhemoglobin and hypoxia (Smith and Fischer, 2001).

Both active (Celermajer et al., 1993; McVeigh et al., 1996) and passive (Celermajer et al., 1996; Kiowski et al., 1994) smoking have been shown to cause impaired endothelium-dependent vasodilation in healthy young adults. Supportive studies using animal models have demonstrated that chronic exposure to ETS is associated with a decrease in vasodilatory function (Hutchison et al., 1997, 1998, 1999; Mays et al., 1999). Acute exposure to second-hand smoke has also been shown to decrease endothelium-dependent vasodilation in humans (Otsuka et al., 2001; Kato et al., 2006), which negatively correlated with levels of oxidative stress in the blood (Kato et al., 2006). Although some studies report that smoking and ETS have no effect on or increases vasodilation (Rangemark and Wennmalm, 1992; Jacobs et al., 1993; Nene et al., 1997; Mudaliar et al., 1997), the majority of investigations have reported that cigarette smoke exposure causes impaired endothelium-dependent vasodilation in humans and animals (Rahman and Laher, 2007).

While endothelial function is tested using flow-mediated dilation in the large brachial artery of humans, endothelial function in the smaller arteries of rodents cannot be tested in this manner. Instead, endothelial function is most often investigated *ex vivo* using isometrically-mounted rodent arteries in tissue baths. Although this *ex vivo* method is great for characterizing functional changes arising in the endothelium and vascular smooth muscle,

the influence of systemic factors carried by the blood are removed. Arterial stiffness may instead be used as a surrogate measure of endothelial function since it is influenced, in part, by vascular smooth muscle tone (Smith et al., 2002; Wilkinson et al., 2002c). Arterial stiffness also allows for measurement with all factors intrinsic and extrinsic to vascular tissues still intact even in small animals. Thus, arterial stiffness may be particularly useful for *in vivo* work in rodent models.

1.7.2 Cigarette Smoke and Arterial Stiffness

Accumulating evidence suggests that changes in the mechanical properties of arteries occur early and may be directly involved in the development and progression of atherosclerosis (Sumitani et al., 1997; Cameron, 1999; Arnett et al., 2000). Recently, interest has focused on arterial stiffness as a surrogate measure of cardiovascular risk and it has been widely investigated in a clinical setting. Acute smoking or ETS exposure has been reported to increase arterial stiffness in humans (Kool et al., 1993; Giannattasio et al., 1994; Stefanadis et al., 1997, 1998; Failla et al., 1997; Mahmud and Feely, 2003, 2004; Argacha et al., 2008). It is well-known that chronic cigarette smoking and ETS cause structural modifications of the arterial wall, thereby reducing arterial distensibility (Rahman and Laher, 2007). Hypertrophy of the arterial wall is an early sign of atherosclerosis. Increases in the intima-media thickness ratio (IMT) or arterial wall thickness are associated with smoking or ETS in both humans and experimental animals (Rahman and Laher, 2007). In humans, smoking is associated with a dose- and/or time-related increase in IMT and impairment in flow-mediated dilation (Rahman and Laher, 2007), while increased IMT is associated with quantifiable increases in arterial stiffness (Mack et al., 2003).

There is also evidence that chronic increases in arterial stiffness occur independent of significant structural changes (Safar et al., 2000; Liang et al., 2001) and that changes in endothelium-dependent vasomotor tone may contribute to chronic reductions in arterial mechanical properties (Safar et al., 2000). In support of this, a recent study reported that the majority of the effect of chronic smoking on arterial stiffness is mediated by endothelial dysfunction (Rehill et al., 2006). A number of studies have also demonstrated that smoking causes deleterious effects on arterial wall mechanics in healthy, young subjects free of other CVD risk factors, including young adult and teenage smokers (Levent et al., 2004; Li et al., 2005) and even children exposed to ETS (Kallio et al., 2009). Furthermore, the acute (Mahmud and Feely, 2003) and chronic (Liang et al., 2001; Vlachopoulos et al., 2004; Li et al., 2005) increases in arterial stiffness following cigarette smoke occur independent of blood pressure changes and may therefore represent active changes in arterial stiffness. Thus, smoking appears to have a direct adverse effect on arterial mechanical properties. Similar to the effects of cigarette smoke, a recent study reported that exposure to diesel exhaust particles, which contains a high concentration of PAHs, increases arterial stiffness in humans (Lundbäck et al., 2009). However, the effect of the PAH component of these contaminants in isolation on arterial stiffness has not yet been studied.

1.7.3 Cigarette Smoke and Vasoconstriction/Hypertension

If active and passive smoking impairs endothelium-dependent vasodilation, then an increased contractile response or increased sensitivity to vasoconstrictive agents would be expected following exposure to cigarette smoke. However, results are equivocal, with reports of increased (Hutchison et al., 1997, 1999; Mays et al., 1999) or decreased (Hutchison et al.,

1998; Simko et al., 2001) maximum contraction to various contractile stimuli in animal models and humans. Smoking causes increases in plasma ET-1 levels in humans (Haak et al., 1994; Goerre et al., 1995) and chronic cigarette smoke exposure in rats has also been shown to increase plasma ET-1 levels and arterial tone (Rahman et al., 2007). It is known that nicotine, either by direct injection or when smoking a cigarette, acutely causes vasoconstriction and increases blood pressure due to stimulation of the adrenal medulla and ganglionic release of norepinephrine and epinephrine (Benowitz, 1986), but the effects of long-term exposure may be different. In a porcine model, chronic exposure to nicotine or water soluble cigarette smoke extract, which contains nicotine, did not affect coronary sensitivity or responsiveness to various constrictor stimuli (Saxon et al., 1984). Thiocyanate, a major end product of cigarette smoking, has been shown to induce contractile responses of rat aorta. However, another study reported that cyanide and nicotine, but not thiocyanate, potentiated the contractile responses of norepinephrine and epinephrine (Tuncel et al., 1994).

In humans, acute smoking or ETS exposure from as little as a single cigarette is associated with a marked increase in blood pressure and heart rate within 5 minutes of exposure (Groppelli et al., 1992; Kool et al., 1993; Failla et al., 1997; Stefanadis et al., 1997, 1998; Mahmud and Feely, 2003). Overall, there is evidence that cigarette smoke acutely increases blood pressure, while both acute and chronic cigarette smoke exposure causes endothelial dysfunction and increases arterial stiffness. In contrast, chronic exposure may not increase the incidence of hypertension in humans (Law et al., 1997; He et al., 1999). In fact, blood pressure has been reported to be, on average, lower in smokers than non-smokers (Green et al., 1986; Goldbourt and Medalie, 1977; Savdie et al., 1984; Benowitz, 1989). The chronic effect of cigarette smoke on blood pressure in experimental animals also varies, with

reports of unchanged (Haag et al., 1960; Barron et al., 1988; Tanaka et al., 2004) or increased blood pressure in rats (Loscutoff et al., 1982) and mice (Guo et al., 2006). Several studies have reported decreased (Green et al., 1991; Mikkelsen et al., 1997) or increased (Mann et al., 1991; Verdecchia et al., 1995; Bolinder and de Faire, 1998; Benowitz et al., 2002; Morillo et al., 2006) ambulatory blood pressure in smokers compared with nonsmokers. However, nocturnal blood pressure dipping is reported to be similar to that of nonsmokers (Morillo et al., 2006). Thus, not all studies are in agreement and the effect of chronic smoking on blood pressure remains elusive (Pickering et al., 1995; Beilin, 2002).

1.7.4 Cigarette Smoke and Cardiac Disease

Chronic smokers are at increased risk of coronary disease and acute myocardial infarction through the development of atherosclerotic lesions (Waters et al., 1996). In addition to atherosclerotic plaque formation, the mechanisms by which smoking increases the prevalence of AMI include thrombosis, endothelial dysfunction, platelet aggregation, arrhythmia induction and vascular spasm (Smith and Fisher, 2001). Cigarette smoking has immediate effects on coronary hemodynamics, causing immediate constriction of coronary arteries and increased vessel tone despite an increase in myocardial oxygen demand. Such acute effects potentially contribute to the adverse cardiovascular consequences of smoking (Quillen et al., 1993) and may occur as a result of increased sympathetic outflow induced by cigarette smoke (Cryer et al., 1976; Narkiewicz et al., 1998) and/or impaired endothelial function in the coronary arteries.

Increased aortic pressure and systemic vascular resistance increases pressure overload and induces cardiac hypertrophic responses. In rats, long-term cigarette smoke exposure has

been reported to cause cardiac structural and functional changes, including ventricular dysfunction, myocardial hypertrophy, ventricular enlargement, and increased aortic diameter (de Paiva et al., 2003; Zornoff et al., 2006; Castardeli et al., 2005, 2008). Reactive oxygen species have been demonstrated to induce functional and structural damage of cardiac myocytes (Ide et al., 1999; Siwik et al., 1999), but antioxidant co-treatment has been reported to prevent cigarette smoke-induced ventricular remodeling in rats (Zornoff et al., 2006). Therefore increased oxidative stress may also play a role in the adverse effects of cigarette smoke on cardiac structure and function.

1.8 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons are extensively metabolized both in the environment and biologically by Phase I and Phase II enzymes as a means of detoxification and elimination from the organism. However, oxidative metabolism of PAHs may lead to the production of highly reactive intermediates, thereby enhancing toxicity of these compounds.

1.8.1 Mechanism of Action

Polycyclic aromatic hydrocarbons are known to exert many of their effects by stimulating the AhR (Nebert et al., 2000; Miller and Ramos, 2001). The AhR controls the expression of several genes, including those which encode xenobiotic-metabolizing enzymes (Nebert et al., 2000). Not only do PAHs increase expression of detoxification enzymes, such as CYP1A1, by activating the AhR, but they are also substrates for these enzymes. These

enzymes catalyze oxidative metabolism of the parent compound into reactive intermediates, which can then interact with cellular macromolecules or generate ROS to elicit toxic effects (Miller and Ramos, 2001).

Several PAHs are procarcinogens that can be metabolically activated to electrophilic metabolites such as epoxides. Electrophilic metabolites form adducts with nucleic acids and proteins, leading to mutagenesis and carcinogenesis and cellular toxicity. Benzo[a]pyrene is the prototypical PAH and possesses a high level of carcinogenicity. Benzo[a]pyrene can be converted to the ultimate carcinogen benzo[a]pyrene 7,8-diol-9,10-epoxide (BPDE), which can form DNA adducts with the N^2 position of guanine (Figure 1.4). Polycyclic aromatic hydrocarbons can also be bioactivated to form quinone structures that are capable of generating large amounts of ROS through redox cycling (Figure 1.5) (Miller and Ramos, 2001). Not only does increased expression of AhR-controlled genes lead to oxidative stress (Nebert et al., 2000), but any subsequent exposure to chemicals such as PAHs would amplify the oxidative stress due to a greater formation of reactive metabolites by the more highly expressed CYP enzymes. Thus, by increasing oxidative stress and the formation of electrophilic metabolites, the PAH component of cigarette smoke could be responsible for the cigarette smoke-associated increase in atherosclerosis. In support of this, smokers have been reported to have increased oxidative stress levels (Reilly et al., 1996), but the role of PAHs in this process is unknown.

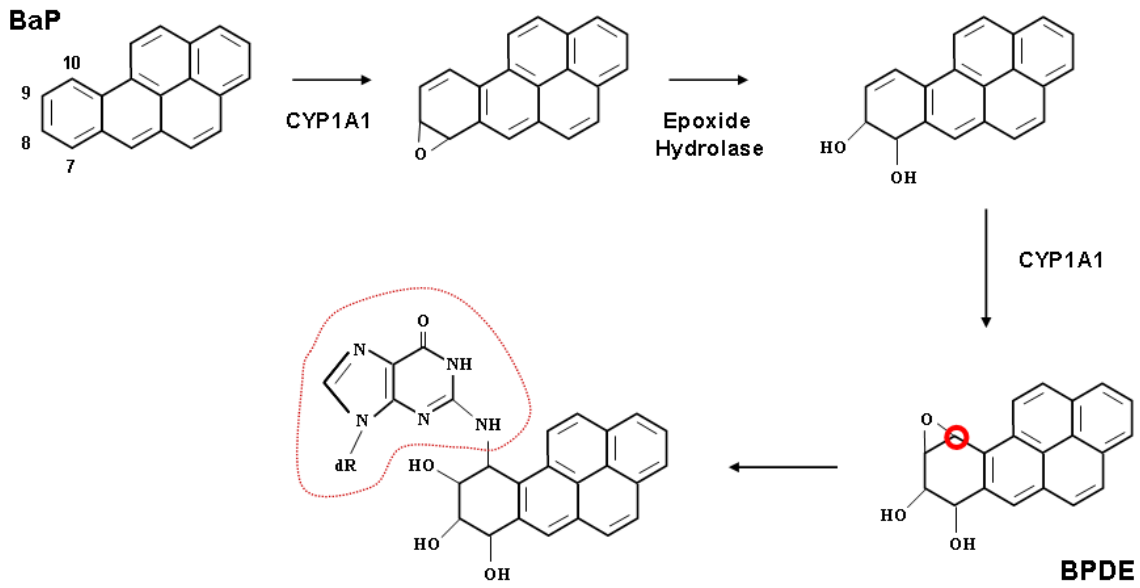


Figure 1.4 Bioactivation of benzo[a]pyrene (BaP) to the ultimate carcinogen, 7,8-diol-9,10-epoxide (BPDE). The first step is oxidation to a 7,8-epoxide catalyzed by cytochrome P450 1A1 (CYP1A1), which is followed by hydrolysis of the epoxide to 7,8-diol by epoxide hydrolase. A second oxidation by CYP1A1 forms the BPDE, which results in covalent adduct formation with the N^2 position of guanine (Miller and Ramos, 2001).

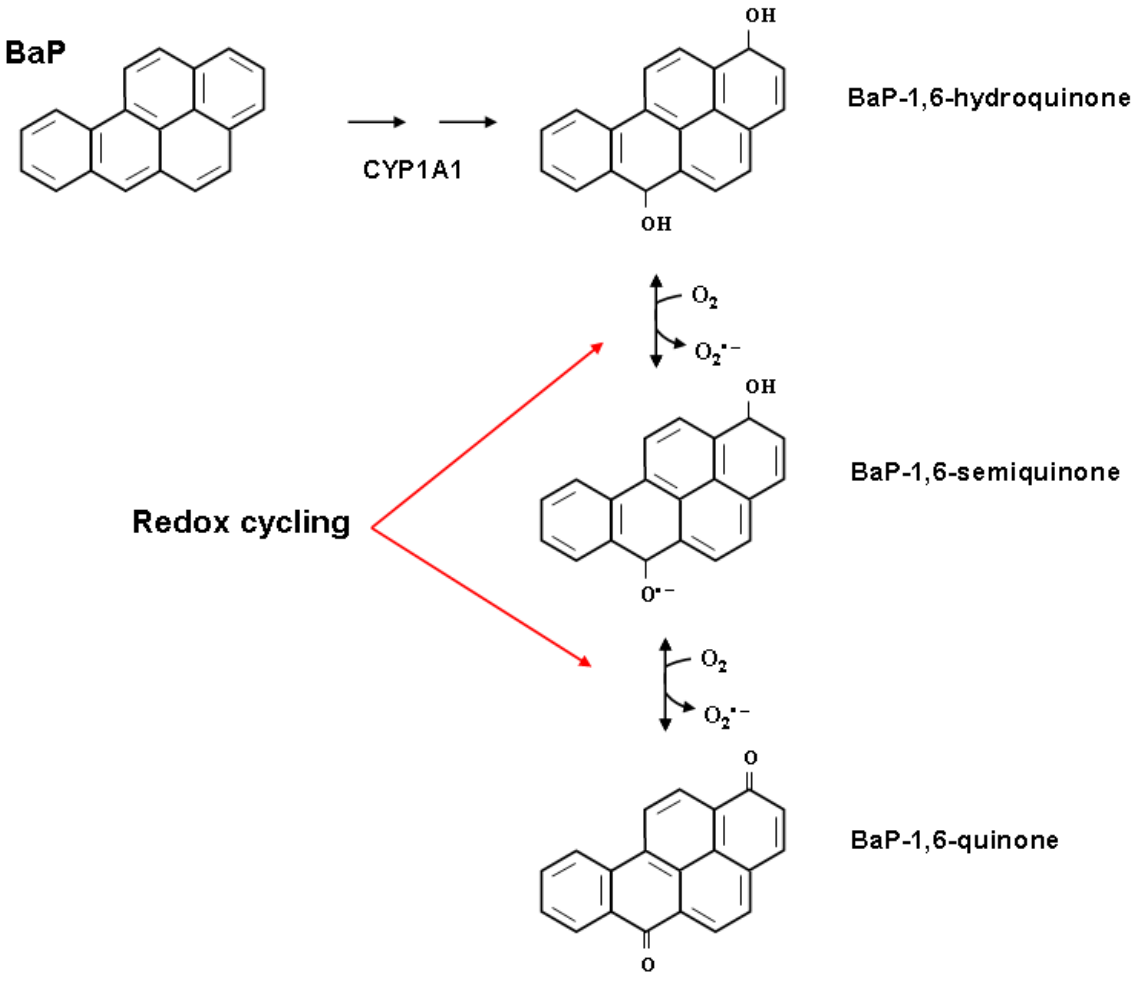


Figure 1.5 Redox cycling of benzo[a]pyrene (BaP) quinone produces reactive oxygen species. BaP is converted to one of its major metabolites, BaP-1,6-hydroquinone, through two cytochrome P450 1A1 (CYP1A1)-catalyzed reactions. This hydroquinone can then undergo auto-oxidation to form BaP-1,6-semiquinone and then BaP-1,6-quinone. Redox cycling of these intermediates results in the generation of large amounts of superoxide anion ($O_2^{\bullet -}$) (Miller and Ramos, 2001).

1.8.2 Polycyclic Aromatic Hydrocarbons and Aryl Hydrocarbon Receptor Ligands in Cigarette Smoke

Polycyclic aromatic hydrocarbons and other AhR ligands are found in the tar particulate phase of cigarette smoke, and exist at a much higher concentrations in sidestream smoke than mainstream smoke (Lofroth and Rannung, 1988; Hoffmann and Hoffmann, 1997). Thus, AhR-mediated effects are particularly relevant to second-hand smoke or ETS. Expression of CYP1A1 is commonly used as a marker of AhR stimulation. Endothelial, lung, and liver cells respond to AhR stimulation with large increases in CYP1A1 expression (Bond et al., 1980, Abraham et al., 1985; Annas and Brittebo, 1998; Kerzee and Ramos, 2001) at environmentally-relevant concentrations (Gebremichael et al., 1996). Although environmental factors such as smoking and genetics independently contribute to the pathogenesis of cancer and vascular diseases, smoking-gene interactions exist. For example, particular polymorphisms in CYP1A1 and eNOS can influence the individual sensitivity to smoking-induced pathogenesis such as atherosclerosis (Wang and Wang, 2005), which suggests key roles for these enzymes in cardiovascular disease.

1.9 The Aryl Hydrocarbon Receptor and Cardiovascular Disease: Clinical and Experimental Studies

1.9.1 The Aryl Hydrocarbon Receptor and Atherosclerosis

Although the typical mechanism of AhR-mediated gene regulation involves binding of the AhR-ARNT transcription factor complexes to DREs, the AhR can regulate transcription through alternate mechanisms. Furthermore, it is known that cross-talk exists between the AhR and other transcription factors (Niermann et al., 2003). Few genes have been shown to be negatively regulated through AhR signaling. T-cadherin is a cell adhesion molecule that is highly expressed in cardiac and vascular tissues and stimulation of the AhR directly causes transcriptional repression of its gene (Niermann et al., 2003). The AhR also represses transcription of transforming growth factor-β and affects the gene expression of several TGF-β-modulating and processing genes (Guo et al., 2004). A recent study identified Niemann–Pick type C1 protein (NPC1) as a molecular target repressed by AhR agonists such as TCDD and BaP, which likely contributes to lipid accumulation in macrophages and the deleterious cardiovascular effects caused by these contaminants (Podechard et al., 2009).

Epidemiological studies indicate that exposure to AhR agonists in the environment, including PAHs (Burstyn et al., 2005) and TCDD (Kim et al., 2003; Pesatori et al., 2003; Kang et al., 2006), promotes the development of atherosclerosis-related cardiovascular disease. Polycyclic aromatic hydrocarbon-DNA adducts have been found in the atherosclerotic lesions of humans, supporting the hypothesis that genotoxic mechanisms may play a role in the development of atherosclerotic plaques (Binkova et al., 2001; Izzotti et al., 1995, 2001; De Flora et al., 1997). In experimental animals, PAHs such as BaP increase the development of atherosclerosis (Albert et al., 1977; Penn and Snyder, 1988; Hough et al., 1993; Curfs et al., 2004, 2005). However, several studies have reported that the atherogenic effect of PAHs were not obviously associated with their mutagenic and carcinogenic capacity (Penn and Snyder, 1988; Curfs et al., 2004, 2005). There is accumulating evidence for non-

genotoxic (epigenetic) mechanisms of atherogenesis by PAHs in experimental animals, including oxidative stress (Wang et al., 2009; Yang et al., 2009) and endothelial adhesiveness (Oesterling et al., 2008). A recent study also reported that *in vitro* BaP exposure increased the expression of matrix metalloproteinases in an AhR-dependent manner, as well as promoted migration and invasion of rat vascular smooth muscle cells (Meng et al., 2009).

Among the genes regulated by the AhR, pro-inflammatory cytokines are an emerging class of importance (N'Diaye et al., 2006). In a recent study, TCDD exposure in mice caused an increase in inflammatory cytokine expression in the lung in an AhR/DRE-dependent manner (Wong et al., 2010). Polychlorinated biphenyls (PCBs) have been shown to increase the expression of adhesion molecules and inflammatory mediators in endothelial cells. There is evidence that this effect is mediated by increased oxidative stress associated with AhR-induced CYP1A1 activity (Slim et al., 1999; Hennig et al., 2002; Choi et al., 2003; Ramadass et al., 2003). PAHs also cause adverse inflammatory effects and have been reported to cause increased expression of various proinflammatory cytokines such as IL- 8 and TNF-α (Lyte and Bick, 1986; Pei et al., 2002; Lecureur et al., 2005; N'Diaye et al., 2006; Knaapen et al., 2007; Podechard et al., 2008) which in turn cause infiltration of macrophages and neutrophils into the lung (Nel et al., 2001; Podechard et al., 2008; Wong et al., 2010). Thus, there is evidence that AhR stimulation, by PAHs or other AhR agonists, increases oxidative stress as well as the expression of inflammatory cytokines and adhesion molecules, all of which are involved in atherogenesis.

1.9.2 The Aryl Hydrocarbon Receptor and Hypertension

Findings from both epidemiological and experimental studies have suggested a role for the AhR in cardiac disease (Heid et al., 2001; Kanzawa et al., 2004; Kang et al., 2006; Korashy and El-Kadi, 2006; Kopf et al., 2008). Recently, BaP (Aboutabl et al., 2009) and TCDD (Kopf et al., 2008) were reported to cause cardiac hypertrophy in rodents, as indicated by increased heart weight to body weight ratio, induction of hypertrophic markers, and/or concentric left ventricular hypertrophy. In humans, TCDD exposure is associated with hypertension (Pesatori et al., 2003; Kim et al., 2003; Kang et al., 2006). In experimental animals, TCDD has also been reported to cause endothelial dysfunction and hypertension (Dalton et al., 2001; Kopf et al., 2008), which is associated with increased superoxide anion formation (Kopf et al., 2008). In contrast, exposure to PAHs in experimental animals has produced equivocal effects on hypertension. In one previous experiment, rats injected with the synthetic PAH, dimethylbenz[a]anthracene, developed hypertension by 3 months of exposure (Kellen and Anderson, 1972). However, another study reported that weekly oral exposure to BaP for 4 weeks did not alter blood pressure (Ichihara et al., 2009). Thus, PAH-induced effects on blood pressure are not clear. In addition, no previous study has examined the effects of BaP on blood pressure after exposure via the airways. Furthermore, the effects of isolated PAHs on arterial stiffness and circadian patterns of blood pressure have not yet been investigated.

1.10 Hypotheses

The following were hypothesized for this thesis:

i. Acute ETS will increase blood pressure and arterial stiffness as a result of decreased NO production and/or bioactivity.
ii. Subchronic exposure to ETS causes endothelial dysfunction, inflammation, oxidative stress, arterial stiffness, hypertension, and atherosclerosis.
iii. Subchronic intranasal administration of BaP will mimic cardiovascular effects of ETS.

1.11 Research Objectives

The following were the objectives for this thesis:

A. Perform acute experiments to validate that pulse wave dP/dt collected from blood pressure telemetry-implanted rats is an indicator of active changes in arterial stiffness as well as examine the effects of acute ETS on blood pressure and arterial stiffness in male Sprague-Dawley rats.
B. Investigate the effects of 28-day ETS exposure on endothelial function, inflammation, oxidative stress, blood pressure, arterial stiffness, cardiac changes, and atherogenesis in rats.

C. Investigate the effects of 7-day intranasal BaP exposure on endothelial function, inflammation, oxidative stress, blood pressure, arterial stiffness, cardiac changes, and atherogenesis in rats.

2.0 USING BLOOD PRESSURE TELEMETRY TO ASSESS ACUTE CHANGES IN ARTERIAL STIFFNESS IN RATS AFTER NITRIC OXIDE SYNTHASE INHIBITION OR ENVIRONMENTAL TOBACCO SMOKE EXPOSURE

2.1 Introduction

Epidemiological studies have established that cigarette smoking and exposure to ETS are major risk factors for cardiovascular disease (Jacobs et al., 1999; Barnoya and Glantz, 2005). Although the underlying pathophysiological mechanisms are still not clear, there is growing evidence for the importance of cigarette smoke effects on the mechanical properties of the arteries (Stefanadis et al., 1998; Mahmud and Feely, 2003). Deterioration in the elastic properties of the arterial wall leads to arterial stiffness, which is defined by a reduction in arterial compliance and distensibility (O'Rourke et al., 2002). Arterial stiffness is a powerful, independent predictor of cardiovascular risk (Blacher et al., 1999, 2000; Laurent et al., 2001) which is influenced by both structural and functional features of the arterial wall (Avolio et al., 1983, 1998; Guerin et al., 2001). Functional regulation of arterial stiffness involves smooth muscle tone, which is influenced by vasoactive mediators such as norepinephrine as well as endothelium-derived NO and ET-1 (Glasser et al., 1997).

Chronic cigarette smoking is known to increase arterial stiffness via structural modifications of the arterial wall (Rahman and Laher, 2007). However, acute smoking or ETS exposure have also been shown to increase arterial stiffness in humans (Kool et al., 1993; Giannattasio et al., 1994; Stefanadis et al., 1997, 1998; Failla et al., 1997; Mahmud and

Feely, 2003; Argacha et al., 2008). A relevant pathway which may explain these effects is an increase in oxidative stress caused by cigarette smoke (Zhang et al., 2002; Huang et al., 2005) and the subsequent biological inactivation of NO. Superoxide anion within the vasculature reacts with NO to form peroxynitrite, which is itself toxic and has been shown to uncouple eNOS. Uncoupled eNOS then produces superoxide anion instead of NO (Münzel et al., 2005), further impairing vasorelaxation and disrupting functional regulation of arterial stiffness. In addition, smoking is known to increase plasma ET-1 levels in humans (Haak et al., 1994; Goerre et al., 1995). Thus, an imbalance between vasorelaxing and contracting factors, such as NO and ET-1, leads to impaired vascular reactivity (De Meyer and Herman, 1997) and may play a key role in the cigarette smoke-induced increases in arterial stiffness.

Pulse wave analysis provides a means for assessing arterial stiffness (O'Rourke et al., 2001). While preclinical studies have used various *in vivo* techniques to assess cardiovascular function in animals, few studies have measured arterial stiffness in rodents after ETS exposure (Liu and Fung, 1993; Guo et al., 2006), none of which were performed *in vivo*. Blood pressure telemetry provides a large amount of reliable, sensitive physiologic data from animals. The pulse wave dP/dt can be easily extracted from arterial pressure waves and we hypothesize that it will increase with arterial stiffening.

Therefore, the present study was designed to first determine whether acute changes in arterial stiffness could be detected following administration of vasoactive drugs acetylcholine (ACh) or norepinephrine (NE) and also after NO synthase inhibition with N(G)-nitro-L-arginine methyl ester (L-NAME). We then examined the effects of acute ETS exposure on blood pressure and dP/dt compared to L-NAME effects. We hypothesize that both L-NAME

and ETS will acutely increase arterial stiffness in rats as a result of decreased NO production (plasma nitrate/nitrite) and/or bioactivity (indicated by increased nitrotyrosine formation).

2.2 Materials and Methods

2.2.1 Animals and Surgery

All protocols were approved by the Animal Care and Use Council at the University of Saskatchewan in accordance with the Canadian Council on Animal Care guidelines. Male Sprague Dawley rats (265-345g) were housed individually under standard conditions with food and water available *ad libitum* except during exposures.

Rats were premedicated with midazolam (0.2 mg/ml i.m.; Sandoz Canada, Inc., Boucherville, CAN) and buprenorphine (20 μg/kg i.m.; Schering-Plough, Hertfordshire, UK). Surgical anaesthesia was initiated at 5% isoflurane (Abbott Laboratories, Saint-Laurent, CAN), then maintained at 3% isoflurane. A PA-C10 radiotelemetry blood pressure transmitter (Data Sciences International, St. Paul, USA) was implanted into the femoral artery and advanced toward the iliac artery with the transmitter body placed subcutaneously in the left flank. Post-operative treatment included thermal support for 1 hr, fluid replacement (5-10 ml saline s.c.), buprenorphine (20 μg/kg i.m. every 12 hr for up to 48 hr), and trimethoprim/sulfadoxine (30 mg/kg i.m. daily; Schering-Plough, Hertfordshire, UK for 7 days). Rats were allowed to recover from surgery for at least 14 days before experiments.

2.2.2 Acute Drug Injection Experiment

Rats were anaesthetized using isoflurane (5% to initiate and 3% to maintain) in order to examine acute effects of cardiovascular drugs using a series of bolus drug injections. Once stable anaesthesia was achieved, arterial pressure was recorded continuously during the entire experiment. A single bolus of saline vehicle (0.5 ml/kg) was injected intravenously via the tail vein (n=4 rats). A washout period of at least 5 minutes was allowed between each injection. Acetylcholine (ACh 0.91 ng/kg; Sigma-Aldrich, St. Louis, USA) was injected, followed by norepinephrine (NE 0.02 mg/kg; Sigma-Aldrich, St. Louis, USA), and finally L-NAME (30 mg/kg; Sigma-Aldrich, St. Louis, USA). Following this drug injection experiment, rats were allowed a minimum of 3 days before sham, then ETS exposures were conducted in the following experiment.

2.2.3 Environmental Tobacco Smoke Experiment

The ETS (mainstream plus sidestream smoke) was generated with a single cigarette manual smoking machine from CH Technologies Inc. (Westwood, USA) at a rate of 3 puff/min (57 ml/puff, 2-s duration). The ETS was mixed with indoor air and pumped into an 89.5 L (low ETS exposure) or 17.3 L (high ETS exposure) inhalation chamber. Pumps controlling inflow and outflow were both set at 6 L/min. Unrestrained, unsedated animals were exposed to ETS from 3 (low ETS) or 5 (high ETS) regular-sized cigarettes (Canadian Classics, Rothmans, Benson and Hedges, CAN) during a one hour exposure period. Sham-exposed rats were placed in identical clean exposure chambers under the same conditions except clean, unfiltered room air was pumped through an unlit cigarette into the chambers for

one hour. All arterial pressure readings were recorded immediately before and after sham or ETS exposures (continuous sampling of blood pressure data for a minimum of 5 minutes).

Exposure conditions were assessed in preliminary experiments by monitoring the total particulate concentrations in the chambers for sham and ETS exposures without rats using a SKC constant airflow pump (Universal 224-PCXR4, Eighty Four, PA) fitted with pre-weighed mixed cellulose ester filters (0.8 μm, SKC Inc., Eighty Four, USA). Particulates were sampled continuously at 2 L/min for one hour (sham), 20 minutes (low ETS), or 12 minutes (high ETS) and expressed as total particulate matter for a one hour exposure period. Carbon monoxide (CO) levels produced during exposure were also measured by placing a T40 Rattler CO monitor (Industrial Scientific, Corp., Oakdale, USA) within the chamber without rats during preliminary experiments. Both temperature and carbon monoxide were measured every 30 seconds for a total of one hour (sham), 20 minutes (low ETS) or 12 minutes (high ETS) and are expressed as the mean for a one hour exposure. Separate preliminary experiments to assess O_2/CO_2 air levels were conducted with a rat in the exposure chamber during a sham exposure using a Criticare Poet IQ multiparameter gas monitor (Criticare Systems, Inc., Waukesha, USA). CO_2 levels remained <1% and mean O_2 levels were 22% (n=3 determinations) during exposures.

2.2.4 Plasma Nitrate/Nitrite, Endothelin-1, Cotinine, and Nitrotyrosine Quantitation

Blood samples were collected at 30 minutes after vehicle/L-NAME injection or sham/ETS exposure. Plasma nitrate/nitrite (NOx; the stable end products of NO) levels were

measured using a commercially available enzyme-based kit (Nitric Oxide Quantitation Kit, Active Motif North America, Carlsbad, USA). Plasma ET-1 (R&D Systems, Inc, Minneapolis, USA), cotinine (Bio-Quant, Inc, San Diego, USA), and nitrotyrosine (Cell Sciences, Canton, USA) levels were quantitated using commercially available enzyme-linked immunosorbent assays.

2.2.5 Data Evaluation and Statistical Analysis

Blood pressure values were averaged from the time encompassing the peak effect of each drug: 30 seconds (ACh) or 1 min (vehicle, NE, and L-NAME). For the ETS experiment, blood pressure was recorded within 5 minutes after exposure and a 2 min segment of data was used for both sham and ETS groups. Values for dP/dt were extracted from arterial pressure waves and averaged in 30 second segments. The segments with peak effects were used for statistical analyses. In order to remove effects of variation among individuals in baseline blood pressure values, pre-exposure blood pressure and dP/dt values were subtracted from post-exposure values from the same individual to determine the treatment effects. All data are expressed as mean ± standard error of the mean (SEM). Differences among 2 treatment groups (sham and ETS) were detected using unpaired, two-tailed t-tests. Differences among multiple treatment groups (vehicle, Ach, NE, L-NAME) were detected using one-way analysis of variance (ANOVA) followed by Tukey's posteriori tests as appropriate. $P<0.05$ was considered to be statistically significant.

2.3 Results

Saline vehicle injections had no significant effect ($p<0.05$ in 1-way ANOVA) on blood pressure, heart rate, or dP/dt compared to pre-injection values (Figure 2.1, Table 2.1). Acetylcholine injection caused a significant decrease in systolic, diastolic and pulse pressures as well as a significant decrease in dP/dt (Figure 2.1, Table 2.1). However, acetylcholine injection caused a significant reflex increase in heart rate (Table 2.1). In contrast, norepinephrine had the opposite effect on all parameters, causing increased pressures, decreased heart rate and increased dP/dt (Figure 2.1, Table 2.1). Injection of L-NAME caused a significant increase in systolic, diastolic, and pulse pressures as well as dP/dt while decreasing heart rate (Figure 2.1, Table 2.1) compared to vehicle control. Interestingly, all blood pressure parameters reached a maximum increase approximately 0.5 minutes after L-NAME injection (Figure 2.2), while the increase in dP/dt did not reach maximum until approximately 1 minute. The maximum decrease in heart rate lagged even further behind, taking closer to 2 minutes to reach a plateau (Figure 2.2). It is important to note that the dP/dt increase was transient while changes in blood pressure and heart rate were sustained for the entire time (at least 5 minutes) recorded after L-NAME injections (Figure 2.2).

ETS exposure had higher total suspended particulates and carbon monoxide levels compared to sham exposure (Table 2.2). Plasma cotinine levels of ETS-exposed rats were also significantly higher compared to sham-exposed rats at 30 minutes after exposure. Average blood pressure and heart rate after sham exposure were 128 ± 3 mmHg (systolic), 88 ± 2 mmHg (diastolic), and 391 ± 19 beats per minute (bpm) (n=4 rats). Compared to sham exposure, ETS-exposed rats showed significant, dose-dependent increases in heart rate and

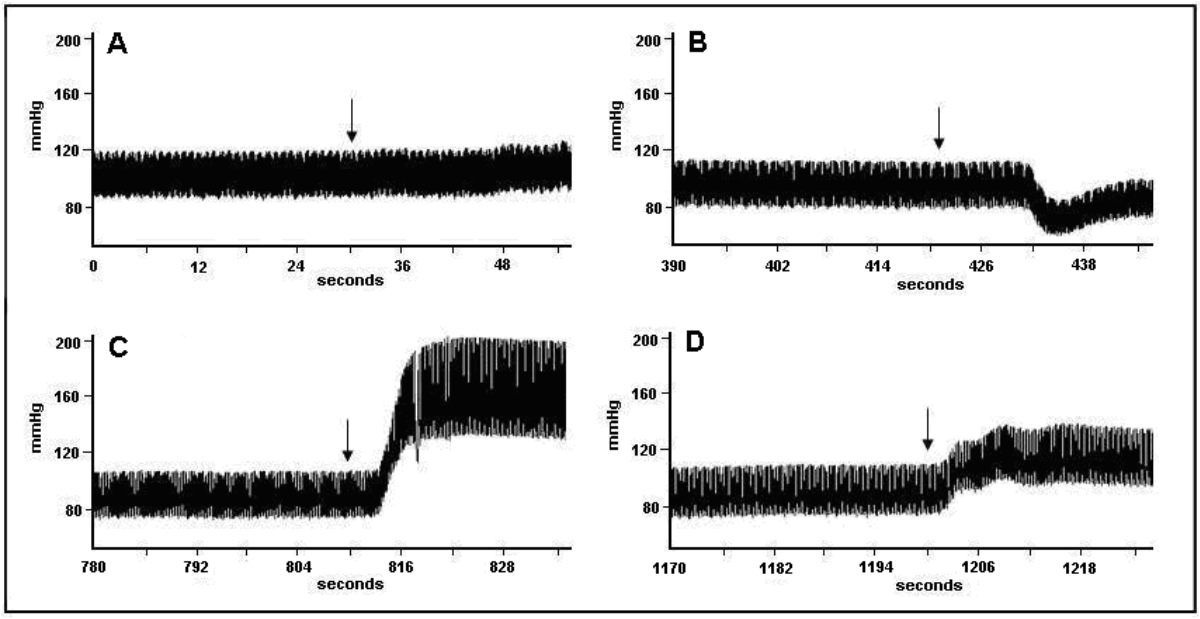


Figure 2.1 Representative blood pressure traces (mmHg) after bolus intravenous administration of A: saline (vehicle control; 0.5 ml/kg), B: acetylcholine (0.91 ng/kg), C: norepinephrine (0.02 mg/kg), and D: N(G)-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg) via the tail vein. Rats (n=4) implanted with radiotelemetry blood pressure transmitters were anaesthetized and injected with saline and then drugs, with a washout period of 5 minutes between each injection. Arrows indicate the time of injection.

Table 2.1 Effects of acetylcholine (ACh), norepinephrine (NE), and N(G)-nitro-L-arginine methyl ester (L-NAME) on blood pressure, heart rate, and arterial pulse wave dP/dt. Rats (n=4) implanted with radiotelemetry blood pressure transmitters were anaesthetized and administered a bolus intravenous injection of 0.5 ml/kg saline vehicle, 0.91 ng/kg ACh, 0.02 mg/kg NE, and then 30 mg/kg L-NAME via the tail vein, with a washout period of 5 minutes between injections. Blood pressure, heart rate (in beats per minute or bpm) and dP/dt values were expressed as the change from individual pre-injection values and are shown as mean ± standard error of the mean. *$p<0.05$, **$p<0.01$ versus vehicle group in Tukey's posteriori test after 1-way analysis of variance.

	Δ Systolic Pressure (mmHg)	**Δ Diastolic Pressure (mmHg)**	**Δ Pulse Pressure (mmHg)**	**Δ Heart Rate (bpm)**	**Δ dP/dt (mmHg/sec)**
Vehicle	-0.7 ± 2.0	0.1 ± 2.0	0.6 ± 0.4	-3 ± 2	35 ± 22
ACh	-16 ± 1**	-11 ± 1**	-6.0 ± 0.7**	4 ± 1*	-460 ± 34**
NE	74 ± 7**	45 ± 5**	30 ± 4**	-23 ± 8*	1340 ± 195**
L-NAME	20 ± 4**	18 ± 2**	2 ± 2	-20 ± 6*	355 ± 144*

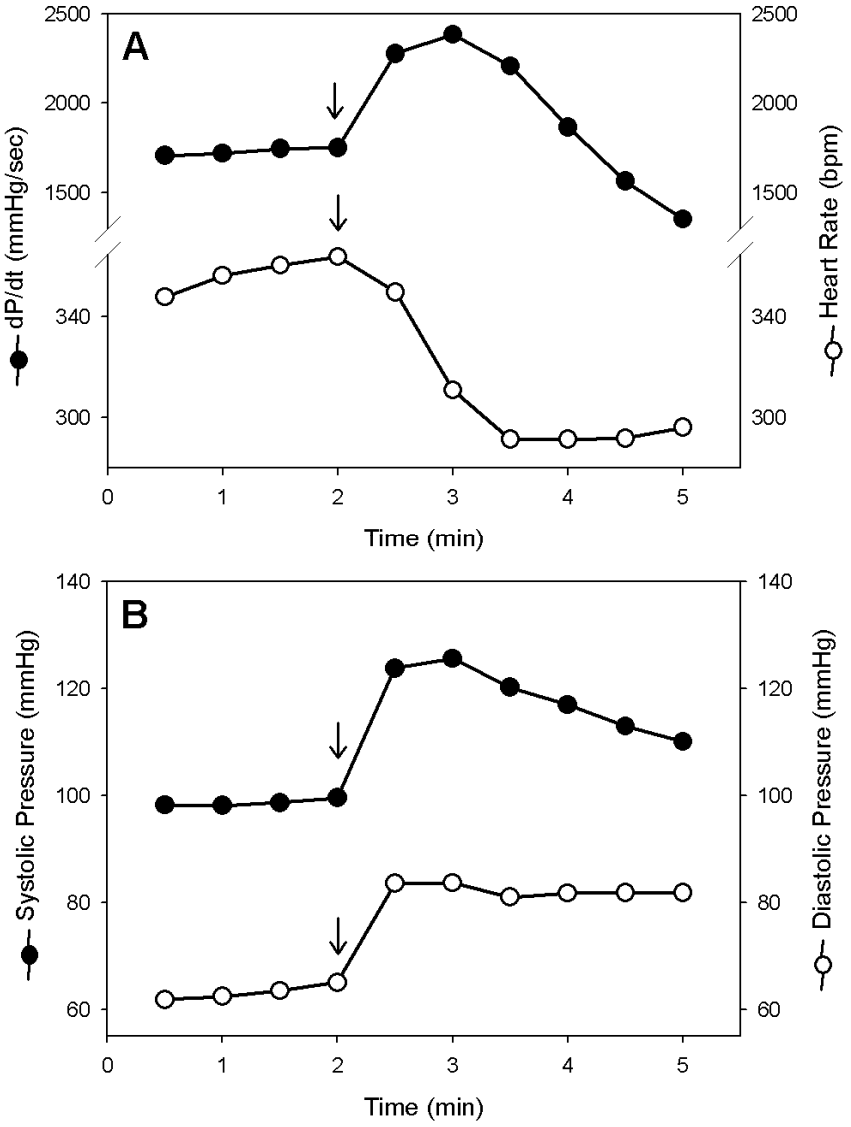


Figure 2.2 Representative time course of change in A: arterial pulse wave dP/dt (filled circles) and heart rate (beats per minute or bpm; open circles) and B: systolic (filled circles) and diastolic pressures (unfilled circles) after bolus intravenous administration of 30 mg/kg N(G)-nitro-L-arginine methyl ester (L-NAME) via the tail vein in blood pressure telemetry implanted rats. Arrows indicate time of L-NAME injection.

Table 2.2 Acute environmental tobacco smoke (ETS) exposure conditions and plasma cotinine concentrations. Conditions in the exposure chamber were determined in the absence of rats in separate preliminary experiments. Rats were exposed to sham (n=4), low ETS (3 cigarettes; n=4) or high ETS (5 cigarettes; n=4) for one hour. Blood samples were collected within 30 minutes after the exposure for plasma cotinine quantitation and data is shown as mean ± standard error of the mean. *p<0.0001 versus sham-exposed group in unpaired *t*-test.

	Sham	**Low ETS**	**High ETS**
Temperature	26ºC	26ºC	25ºC
CO	0	358 ppm	>600 ppm
Particulate Matter	5 $\mu g/m^3$	360 $\mu g/m^3$	1260 $\mu g/m^3$
Plasma Cotinine	0.08 ± 0.03 ng/ml	30.3 ± 3.8 ng/ml*	39.5 ± 5.0 ng/ml*

decreases in pulse pressure (Table 2.3). Diastolic pressure was not significantly altered compared to sham exposure at either ETS exposure level. However, high ETS showed a significant decrease in systolic blood pressures while low ETS exposure was not significantly changed compared to sham exposure.

L-NAME injection caused a significant increase in dP/dt that was accompanied by a significant decrease in plasma nitrate/nitrite compared to vehicle injection (Figure 2.3A). In contrast, ETS at both low and high exposures failed to significantly alter dP/dt compared to sham exposure (Figures 2.3B and 2.3C). However, the high ETS, but not low ETS exposure caused a significant increase in plasma nitrate/nitrite levels (Figure 2.3B and 2.3C). Finally, both low and high ETS exposures caused significant increase in plasma nitrotyrosine while ET-1 levels were not significantly altered (Figure 2.4).

2.4 Discussion

One of the findings of the current study is that pulse wave analysis from blood pressure telemetry-implanted rats may be useful as an indicator of acute changes in arterial stiffness in rats. The dP/dt of the arterial pressure pulse waves was increased by norepinephrine and L-NAME injection, but decreased by acetylcholine injection. The L-NAME change in dP/dt occurred in a different time course than blood pressure and heart rate changes, suggesting it was independent of these parameters. More importantly, although ETS exposure caused increases in peroxynitrate formation, there was a concomitant increase in NO production that may account for the lack of acute ETS-mediated change in arterial stiffness.

Table 2.3 Effects of acute environmental tobacco smoke (ETS) exposure on blood pressure and heart rate in blood pressure telemetry-implanted rats. Rats were exposed to sham (unlit cigarette; n=4 rats), low ETS (3 cigarettes; n=4) or high ETS (5 cigarettes; n=4) for one hour. Blood pressure and heart rate (in beats per minute or bpm) are expressed as the change from individual pre-injection values and are shown as mean ± standard error of the mean. *p<0.05 versus sham-exposed group in unpaired *t*-test.

	Δ Systolic Pressure (mmHg)	**Δ Diastolic Pressure (mmHg)**	**Δ Pulse Pressure (mmHg)**	**Δ Heart Rate (bpm)**
Sham	3 ± 2	2 ± 2	1 ± 1	18 ± 9
Low ETS	1 ± 3	4 ± 2	-2 ± 1*	97 ± 19*
Sham	10 ± 3	9 ± 3	3 ± 1	56 ± 19
High ETS	-7 ± 6*	-2 ± 4	-5 ± 2*	125 ± 18*

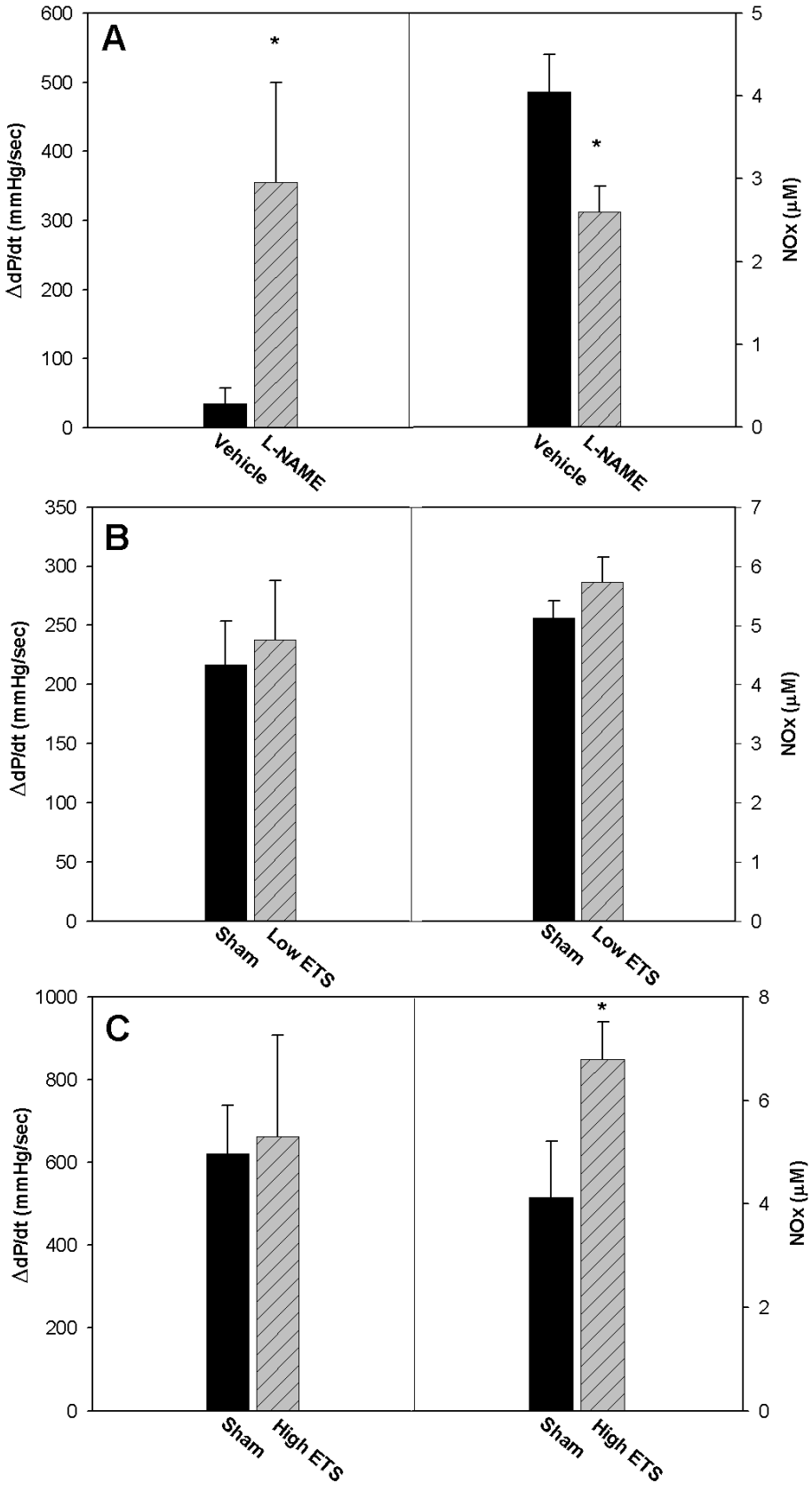

A
ΔdP/dt (mmHg/sec)
NOx (μM)
Vehicle
L-NAME
B
Sham
Low ETS
C
High ETS

Figure 2.3 Effects of A: N(G)-nitro-L-arginine methyl ester (L-NAME) and B: low or C: high environmental tobacco smoke (ETS) on arterial pulse wave dP/dt and on plasma nitrate/nitrite (NOx) levels. Rats implanted with radiotelemetry blood pressure transmitters (n=4) were anaesthetized and administered a bolus dose of saline (vehicle control; 0.5 ml/kg; solid bars) followed by a washout period of 5 minutes and then 30 mg/kg L-NAME (hatched bars) via the tail vein. For the ETS experiment rats were exposed to sham (unlit cigarette; n=4; solid bars), low ETS (3 cigarettes; n=4; hatched bars) or high ETS (5 cigarettes; n=4; hatched bars) for one hour. Values for dP/dt were expressed as the change from individual pre-injection values and plotted as mean ± standard error of the mean. *$p<0.05$ versus sham-exposed group or vehicle group in unpaired *t*-test or 1-way analysis of variance, respectively.

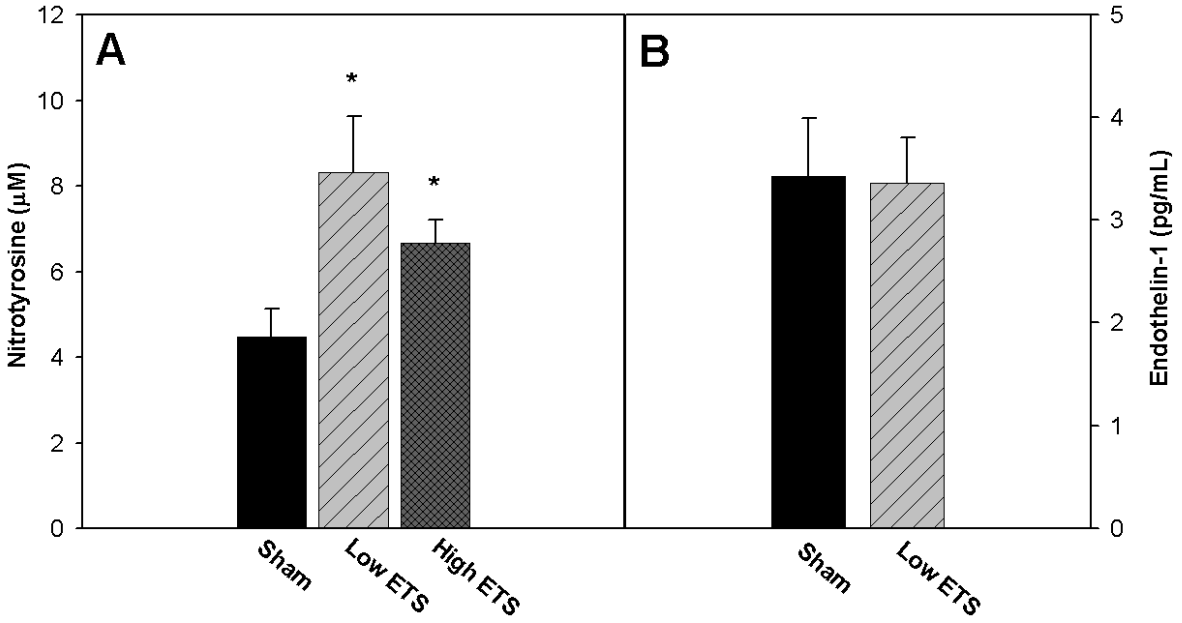


Figure 2.4 Effect of low or high environmental tobacco smoke (ETS) on A: plasma nitrotyrosine and B: endothelin-1 (ET-1) levels. Rats implanted with radiotelemetry blood pressure transmitters exposed to sham (unlit cigarette; n=4 rats), low ETS (3 cigarettes; n=4) or high ETS (5 cigarettes; n=4) for one hour. Data are expressed as mean ± standard error of the mean. *$p<0.05$ versus sham-exposed group in unpaired *t*-test.

Smooth muscle tone is an important determinant of stiffness in the muscular arteries (Nichols, 2005). Changes in arterial stiffness as a result of altered smooth muscle tone are considered an intrinsic or active change in arterial elastic properties (Stefanadis et al., 1998). Arterial stiffness is also acutely influenced by transmural distending pressure, but these changes are passive (Nye, 1964; Stefanadis et al., 1998). Thus, if the intrinsic arterial elastic properties do not change, then an increase in pulse pressure alone (at a constant heart rate) could increase pulse wave dP/dt and cause apparent increases in arterial stiffness. A change in heart rate (at a constant pulse pressure) may also appear to influence arterial stiffness by increasing or decreasing the time for distension (Bergel, 1961; Mangoni et al., 1996). Conversely, if both heart rate and pulse pressure are constant, active changes in arterial elastic properties would increase dP/dt and systolic pressure without affecting or possibly even decreasing diastolic pressure. This arises because of altered pulse wave velocity and subsequent pressure from reflected pressure waves adding to the systolic rather than diastolic phase (O'Rourke et al., 2002).

In the current study, both blood pressure and arterial pulse wave dP/dt increased with norepinephrine and decreased with acetylcholine injection, consistent with expected changes in arterial stiffness. However, as discussed above, passive changes must first be considered before this can be attributed solely to changes in arterial stiffness. For example, norepinephrine injection caused a 30 mmHg increase in pulse pressure in the current experiment, which was calculated to contribute only 161 mmHg/sec to the observed increase of 1340 mmHg/sec in pulse wave dP/dt. In contrast, the observed decrease in heart rate after norepinephrine would have counteracted the observed increase in dP/dt. Therefore, since the observed magnitude of dP/dt change was approximately 10 times higher than the passive

change produced by the pulse pressure increase, the majority of the dP/dt change must be due to active increases in arterial stiffness after norepinephrine administration. In a similar line of argument with acetylcholine, the observed decrease in dP/dt (-460 mmHg/sec) was more than 10 times larger than that which could have been caused by the acetylcholine-induced decrease in pulse pressure (calculated to be -34 mmHg/sec). Again, the change in heart rate would have counteracted the observed effect of acetylcholine on pulse wave dP/dt. Therefore, based on the results of the current experiments with norepinephrine and acetylcholine, changes in dP/dt appear to truly represent active changes in arterial stiffness as a result of alterations in smooth muscle tone.

The changes in dP/dt induced by acetylcholine are known to be largely due to endothelium-dependent release of NO. It is well known that NO contributes to blood pressure regulation (Ignarro et al., 1987). Inhibition of NO synthesis has been shown to acutely increase blood pressure (Rees et al., 1989) and arterial stiffness in humans (Kinlay et al., 2001; Wilkinson et al., 2002a) and animals (Wilkinson et al., 2002b; Fitch et al., 2001). In the current study, NO production was confirmed to be decreased (decreased NOx) after L-NAME administration. The passive changes produced by the observed reduction in heart rate after L-NAME treatment would have reduced dP/dt, while pulse pressure was unchanged. Therefore, passive changes do not account for the 355 mmHg/sec increase in dP/dt observed in the current study after L-NAME administration, consistent with active changes in arterial stiffness. Furthermore, since the changes in systolic and diastolic pressures occurred in a different time course than dP/dt and heart rate changes, this lends further support to the fact that dP/dt of the pulse wave is measuring a true, active change in arterial stiffness. A compensatory decrease in heart rate that followed the increase in dP/dt likely masked the

continuing arterial stiffness induced by L-NAME in the current experiment. Thus, dP/dt and pulse wave analyses appear to be useful in monitoring active changes in arterial stiffness in response to a variety of vasoactive agents.

Acute smoking and ETS have also been reported to increase arterial stiffness in both the medium-sized muscular arteries as well as large elastic arteries (Kool et al., 1993; Giannattasio et al., 1994; Stefanadis et al., 1997, 1998; Failla et al., 1997; Mahmud and Feely, 2003; Argacha et al., 2008). The increased arterial stiffness could be attributed, at least in part, to endothelial dysfunction, increased ET-1, or reduced NO bioactivity in the vasculature. Although smoking has been reported to increase plasma ET-1 in humans, (Haak et al., 1994; Goerre et al., 1995), acute ETS exposure in the current study did not significantly alter plasma ET-1 in rats. Studies have shown that impaired endothelial function, as assessed by flow-mediated dilation, is associated with increased arterial stiffness (Jadhav and Kadam, 2005; McEniery et al., 2006). Indeed, acute ETS exposure has also been shown to impair flow-mediated vasodilation in humans (Otsuka et al., 2001; Kato et al., 2006) and this correlated with levels of oxidative stress in the blood (Kato et al., 2006). Furthermore, plasma NOx levels have been reported to decrease in humans after smoking a single cigarette (Tsuchiya et al., 2002), while the current acute study and several chronic animal studies have reported increased NOx levels with enhanced vasodilation (Alving et al., 1992; Nene et al., 1997). The results of the current study support our hypothesis that acute ETS exposure would increase oxidative stress and decrease NO bioactivity. However, contrary to our hypothesis, ETS exposure did not significantly alter arterial stiffness in the current study as indicated by arterial pulse wave dP/dt. Since the high ETS exposure also had increased plasma NOx levels, there may have been a sufficient increase in NO production to

compensate for the observed NO inactivation, possibly explaining the lack of change in arterial stiffness. Alternatively, cigarette smoke itself can serve as an exogenous source of NO (Hoffman and Hoffman, 1997) which may have acted on the vasculature to prevent decreases in vascular compliance. However, given the short half-life of the NO free radical, it seems unlikely that NO inhaled from ETS would have an effect beyond the lungs. An alternative explanation is that some other, more stable component of ETS is responsible for causing alterations in NO production and bioactivity within the peripheral vasculature of ETS-exposed rats.

Of the thousands of chemicals in cigarette smoke, nicotine is thought to be largely responsible for the acute pressor and tachycardic responses to cigarette smoke in humans, mainly via activation of the sympathetic nervous system and catecholamine release (Benowitz, 1986). There is also evidence that nicotine may also play a role in the acute increase in arterial stiffness after cigarette smoke exposure in humans (Argacha et al., 2008; Adamopoulous et al., 2009). In the current study, since sham exposure increased blood pressure and heart rate, the stress from confinement in the exposure chamber in conscious, unsedated rats and subsequent sympathetic activation may have exerted a larger effect on blood pressure and dP/dt than ETS itself. Also, a transient increase in dP/dt may have been missed in the current study since blood pressure was not recorded until a few minutes after the exposure had ended.

In humans, acute smoking or ETS exposure has been reported to increase blood pressure and heart rate (Groppelli et al., 1992; Kool et al., 1993; Failla et al., 1997; Stefanadis et al., 1998; Mahmud and Feely, 2003) for approximately 15 minutes (Mahmud and Feely, 2003) or have no effect (Argacha et al., 2008). In contrast, results in experimental

animals are varied, with reports of decreased blood pressure in guinea pigs (Wright and Harrison, 1990) and increased (Houdi et al., 1995) or unchanged (Tanaka et al., 2004) blood pressure in rats. In the current study, low ETS exposure had no significant effect on blood pressure while high ETS exposure decreased blood pressure. Levels of cotinine (a nicotine metabolite), air particulates and chamber CO were increased dose-dependently compared to sham in the current experiment. The plasma cotinine levels in the current study fell between those detected in smokers and long-term ETS-exposed humans (Benowitz, 1999). Also, the particulate matter concentrations in the current study are comparable to other laboratory animal ETS studies (Ayres et al., 2001; Chen et al., 2008), suggesting that exposure conditions were relevant. However, the carbon monoxide may explain the observed decrease in blood pressure since it is known to cause vasodilation through its direct effect on vascular smooth muscle cells (Morita et al., 1995; Jaggar et al., 2002) or indirectly through the formation of carboxyhemoglobin and hypoxia (Smith and Fischer, 2001). The carbon monoxide concentration of inhaled cigarette smoke may reach as high as 500 ppm (WHO, 1999) which agrees with the range found in the current study. However, exposure to carbon monoxide alone at concentrations greater than 1000 ppm has been shown to have no effect on blood pressure in humans (Hausberg and Somers, 1997; Zevin et al., 2001). The current ETS experiment measured intravascular blood pressure using telemetry in conscious, unanaesthetized rats while all previous studies have either measured blood pressure indirectly (human and some animal studies) or have used some form of anaesthetic/sedative (most animal studies). With the use of more sensitive methods, we conclude that the effect of ETS on rodent blood pressure, if any, is small or transient compared to that reported previously in humans.

In conclusion, we have demonstrated that acute changes in arterial stiffness could be detected in radiotelemetry-implanted rats using arterial pulse wave dP/dt. Furthermore, increased arterial stiffness after inhibition of NO synthesis is associated with decreased NO. Although acute ETS did not significantly alter blood pressure or arterial stiffness in rats, the apparent increase in reactive nitrogen species production may have been counteracted sufficiently by increased endogenous NO production, thereby preventing acute changes in vascular compliance. However, contributions from stress or ETS-induced release of vasoconstrictive factors cannot be ruled out and will be the focus of future investigations.

3.0 ARTERIAL STIFFNESS AND ALTERED CIRCADIAN BLOOD PRESSURE PATTERNS FROM ENVIRONMENTAL TOBACCO SMOKE EXPOSURE IN RATS IS ASSOCIATED WITH LUNG INFLAMMATION AND OXIDATIVE STRESS

3.1 Introduction

Substantial epidemiological evidence indicates that smoking and ETS exposure are major risk factors for cardiovascular disease (Jacobs et al., 1999; Barnoya and Glantz, 2005). Many of the traditional risk factors for CVD, including smoking, adversely alter endothelial function as well as the structure and mechanical properties of the arterial wall (Gibbons and Dzau, 1994). Arterial stiffness is a powerful, independent predictor of cardiovascular risk (Blacher et al., 1999, 2000; Laurent et al., 2001) and is influenced by both structural and functional features of the arterial wall (Avolio et al., 1983, 1998; Guerin et al., 2001).

Functional regulation of arterial stiffness involves smooth muscle tone (Guerin et al., 2001) which is influenced by both local and circulating mediators (Glasser et al., 1997). Endothelium-derived NO and ET-1 function as mutual antagonists in the determination of vascular tone and several lines of evidence indicate that they play a direct role in the regulation of arterial stiffness (Fitch et al., 2001; Kinlay et al., 2001; Wilkinson et al., 2002a, 2000b; McEniery et al., 2003). In addition, NO also limits several other processes which are associated with atherogenesis, including platelet activation, leukocyte adhesion to the vascular wall, and smooth muscle cell proliferation (Schulz et al., 2004). Thus, an imbalance between contracting and relaxing factors such as NO and ET-1, respectively, may contribute

to endothelial dysfunction (Rubanyi, 1993) and play a key role in the development of atherosclerosis and CVD.

Oxidative stress is an important pathway by which NO levels may be reduced in the vasculature. Superoxide anion quickly reacts with NO to form the highly reactive peroxynitrite, biologically inactivating NO before it can mediate smooth muscle relaxation and disrupting functional regulation of arterial stiffness (Beckman, 1996). Thus, not only have the vasoprotective actions of NO been lost, but peroxynitrite also uncouples eNOS, which then produces superoxide anion instead of NO (Münzel et al., 2005), further shifting the system towards oxidative pathophysiology and proatherogenic milieu.

Cigarette smoke contains a high concentration of ROS and other free radicals as well as stable components which have the potential to increase intracellular production of ROS (Pryor et al., 1993). For example, PAHs can transcriptionally induce phase I and II metabolic enzymes such as CYP1A1/1A2 through activation of the AhR. Polycyclic aromatic hydrocarbons are also substrates for these enzymes and are metabolically activated by CYP1A1 to form quinone structures which then undergo redox cycling to generate large amounts of superoxide anion (Miller and Ramos, 2001). The inflammatory effects of cigarette smoke are also well-known (van der Vaart et al., 2004; Rahman and Laher, 2007) and may also serve as a source of oxidative stress. Activated neutrophils and macrophages can generate ROS (Rennard and Daughton, 1993) and release inflammatory cytokines, inducing the production of ROS in other cells including vascular smooth muscle cells (De Keulenaer et al., 1998). We hypothesize that CYP1A1 induction and inflammatory reactions in the lung following ETS exposure are sufficient to increase oxidative stress in the peripheral circulation, thereby affecting endothelial function.

Pulse wave analysis provides a means for assessing arterial stiffness (Chapter 2; O'Rourke et al., 2001). Chronic smoking is known to cause structural modifications of the arterial wall, thereby reducing arterial elastic properties (Rahman and Laher, 2007). However, recent studies have reported that chronic smoking and ETS increase arterial stiffness even in the absence of atherosclerosis of the vessel (Liang et al., 2001; Li et al., 2005; Kallio et al., 2009). While preclinical studies have used various *in vivo* techniques to assess cardiovascular function in animals, few studies have measured arterial stiffness in rodents after ETS exposure (Liu and Fung, 1993; Guo et al., 2006) and none of these studies were performed *in vivo.* It is a paradox that while smoking increases arterial stiffness, epidemiological studies have generally shown that the blood pressure of smokers is lower than that of nonsmokers (Green et al., 1986). A previous study from this laboratory examining the acute effects of ETS in rodents support this observation, since ETS either had no effect or decreased blood pressure (see Chapter 2). Although ambulatory blood pressure measurements have helped somewhat in clarifying this paradox, not all studies are in agreement and the effect of chronic smoking on blood pressure remains elusive (Beilin, 2002).

We hypothesize ETS will increase ET-1 and that oxidative stress arising from lung inflammation and/or reactive metabolites will reduce NO bioactivity, leading to increased arterial stiffness and blood pressure. We have previously demonstrated that pulse wave dP/dt collected from blood pressure telemetry-implanted rats is a reliable indicator of acute, active changes in arterial stiffness in rats (see Chapter 2). Therefore the objective of this study was to determine whether daily ETS exposure for 28 days would induce changes in blood pressure and arterial stiffness in rats. A second objective was to examine whether any

changes were related to oxidative stress, inflammation, and/or CYP1A1 activity in the lung. In addition, structural (wall thickness) and functional (ET-1, NO production and bioactivity) features of the arterial wall were examined.

3.2 Materials and Methods

3.2.1 Animals and Surgery

Animals and surgery, exposure to ETS, and measurement of plasma cotinine, NOx, ET-1, and nitrotyrosine were conducted as described elsewhere (Chapter 2). Briefly, protocols were approved by the Animal Care and Use Council at the University of Saskatchewan in accordance with the Canadian Council on Animal Care guidelines. Male rats (195-225g) were housed individually under standard conditions with food and water available *ad libitum* except during exposures.

Rats (n=4 per treatment group) were pretreated with midazolam (0.2 mg/ml i.m.; Sandoz Canada, Inc., Boucherville, CAN) and buprenorphine (20 μg/kg i.m.; Schering-Plough, Hertfordshire, UK). Surgical anaesthesia was induced at 5% isoflurane (Abbott Laboratories, Saint-Laurent, CAN), then maintained at 3% isoflurane. A PA-C10 radiotelemetry blood pressure transmitter (Data Sciences International, St. Paul, USA) was implanted into the femoral artery and advanced toward the iliac artery with the transmitter body placed subcutaneously in the left flank. Post-operative treatment included thermal support for 1 hr, fluid replacement immediately post-operative (5-10 ml saline s.c.), buprenorphine (20 μg/kg i.m. every 12 hr for up to 48 hr), and trimethoprim/sulfadoxine (30

mg/kg i.m. daily; Schering-Plough, Hertfordshire, UK for 7 days). Rats were allowed to recover from surgery for at least 14 days before experiments.

3.2.2 Environmental Tobacco Smoke Exposure

Environmental tobacco smoke was generated with a single cigarette manual smoking machine from CH Technologies Inc. (Westwood, USA) at a rate of 3 puff/min (57 ml/puff, 2-s duration). The ETS was mixed with indoor air and pumped into a 89.5 L inhalation chamber. Pumps controlling inflow and outflow were both set at 6 L/min. Animals were restrained in individual wire mesh tubes within the chamber and exposed together (n=8) to ETS from 3 regular-sized cigarettes (Canadian Classics, Rothmans, Benson and Hedges, CAN) during a one hour exposure period every day for 28 days. Sham-exposed rats (n=8) were restrained and placed in identical clean exposure chambers under the same conditions except clean, unfiltered room air was pumped into the chambers for one hour every day for 28 days.

In preliminary experiments, exposure conditions were assessed by monitoring the total particulate concentrations in the chambers for sham and ETS exposures without rats using a SKC constant airflow pump (Universal 224-PCXR4, Eighty Four, PA) fitted with pre-weighed mixed cellulose ester filters (0.8 μm, SKC Inc., Eighty Four, USA), run at 2 L/min for 1 hour (sham) or 20 minutes (ETS). Carbon monoxide levels were also measured by placing a T40 Rattler CO monitor (Industrial Scientific, Corp., Oakdale, USA) within the chamber without rats during preliminary experiments. In separate preliminary experiments, O_2/CO_2 air levels were assessed using a Criticare Poet IQ Multiparameter gas monitor (Criticare Systems, Inc., Waukesha, USA) with 8 rats in the exposure chamber. CO_2 levels

remained <1% and mean O_2 levels were 21% (n=3 determinations) during the sham exposures.

3.2.3 Plasma Nitrate/Nitrite, Endothelin-1, Cotinine, and Nitrotyrosine Quantitation

Blood samples were collected at 30 minutes after sham and ETS exposures. Plasma NOx levels were measured using a commercially available enzyme-based kit (Nitric Oxide Quantitation Kit, Active Motif North America, Carlsbad, USA). Plasma ET-1 (R&D Systems, Inc, Minneapolis, USA), cotinine (Bio-Quant, Inc, San Diego, USA), and nitrotyrosine (Cell Sciences, Canton, USA) levels were quantitated using commercially available enzyme-linked immunosorbent assays.

3.2.4 Lung and Liver Ethoxyresorufin-o-deethylase (EROD) Activity

Rat liver and lung were homogenized (PT10135 homogenizer; Brinkmann Instruments Co., Mississauga, Ontario, CAN) in ice-cold 0.05 M Tris (Sigma-Aldrich, St. Louis, USA)/1.15% KCl (EMD Biosciences, San Diego, USA, 99% purity) (pH 7.5) buffer solution. The ratio of tissue to homogenization buffer was 2 g/25 ml. The homogenate was immediately centrifuged at 10,000g at 4 °C for 20 min in an RC5C centrifuge (Sorvall instruments, Mandel Scientific Co. Ltd., Guelph, CAN). Microsomes in the supernatant were then pelleted at 100,000g at 4 °C for 60 min in a Sorvall WX Ultra 80 ultracentrifuge

(Thermo Electron Corp., Waltham USA). The pellet was resuspended in 0.25M sucrose (EMD Biosciences, San Diego, USA, molecular biology grade) and frozen at -80º C, until analysis.

Protein concentrations in liver and lung microsomal preparations were determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Mississauga, CAN) using bovine serum albumin (Sigma-Aldrich, St. Louis, USA) as a calibration standard. For the EROD assay, standards were prepared from resorufin sodium salt (Sigma-Aldrich, St. Louis, USA) diluted with dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA). Beta-nicotinamide adenine dinucleotide phosphate (NADPH Sigma-Aldrich, St. Louis, USA, 94%) was dissolved in water (1.25 mM) just before preparation of the sample series. The reaction was initiated by addition of NADPH to the sample solution. Samples were analyzed in the following assay conditions: pH 7.4 (37°C) for 75 min in a mixture of Hepes buffer (75.5 mM, Sigma-Aldrich, St. Louis, USA, 99.5%) with $MgCl_2$ (3.77 mM, EMD Biosciences, San Diego, USA, molecular biology grade), ethoxyresorufin (5 µM, Sigma-Aldrich, St. Louis, USA, 95%) dissolved in DMSO, 20 µg liver or lung protein, and NADPH (0.25 mM) in a final volume of 250 µl. A positive control sample (liver microsomes prepared from rainbow trout injected 2x with 10 mg/kg benzo[a]pyrene over 72 hrs) was analyzed in every sample series to ensure consistent assay performance.

3.2.5 Histological Analysis

Whole heart, a portion of the lung (lower left lobe), thoracic aorta immediately after the arch, and the abdominal aorta with iliac branches were dissected from rats euthanized at day 29 of treatment (n=8 per treatment group) and transferred into neutral buffered formalin.

After 24 hours, samples were transferred to 70% ethanol and stored until histological analysis could be performed. Samples were paraffin-embedded and cross-sectioned (5 μm, serial sections), then stained with hematoxylin and eosin. For the heart, 3 serial sections were analyzed at 4 mm above the apex. Size analysis of the heart was performed using digital photomicrographs obtained with an Olympus SZ61 dissecting microscope and 5.0 Megapixel Olympus Q-Color5™ Camera System (Olympus Canada Inc., Markham, CAN). Photomicrographs were taken at 11x magnification and analyzed via Image-Pro software. All other analyses utilized digital photomicrographs obtained with a Zeiss AxioVert light microscope. Heart and vascular analysis were photographed at 50x magnification and analyzed using AxioVision software. For the heart, left ventricular wall thickness was measured in 3 serial sections and averaged. For each artery, the average luminal diameter (LD) was averaged from 3 serial sections, being derived from the luminal area: LD = 2*square root(area/π). The outer artery area was similarly used to derive the average outer diameter (OD) in 3 sections per rat. Medial thickness (MT) was then calculated from these values: MT = (OD-LD)/2. For the lung, cross sections were scanned for 4 zones of high neutrophil numbers, where the number of neutrophils was counted at 400x magnification. A single mean value per animal was used for all statistical comparisons.

3.2.6 Data Evaluation and Statistical Analysis

Over the course of the 28 day exposure period, resting blood pressure and heart rate data was collected weekly for 20 minutes before daily exposures began (approximately 22 hours after the previous day's exposure). To obtain the circadian pattern of blood pressure and heart rate, data was collected once a week for a period of 24 consecutive hours (10

minutes of sampling per rat per hour) starting within 2 hours of the end of the daily exposure and ending prior to the exposure periods the next day. In order to minimize effects of variation among individuals in baseline blood pressure values and determine treatment effects, pre-exposure (baseline or day 0) blood pressure, heart rate, and dP/dt values were subtracted from post-exposure values from the same individual. The percent dip in blood pressure was calculated as the raw blood pressure, heart rate, or dP/dt value during the dark/active period divided by the value during the light/inactive period and multiplied by 100. All data are expressed as mean ± SEM. Differences among groups were detected using unpaired, two-tailed *t*-test (single time point data), two-way (data with factors for week of exposure and treatment), or three-way (data with factors for time of day, week of exposure and treatment) ANOVA followed by Tukey's posteriori tests as appropriate. However, all organ weights and tissue sizes were analyzed by analysis of covariance (ANCOVA) with body weight as a covariate.

3.3 Results

The ETS exposure had higher total suspended particulates and carbon monoxide levels compared to sham exposure (Table 3.1). Plasma cotinine levels of ETS-exposed rats were also significantly higher compared to sham-exposed rats at 30 minutes after exposure. In liver and lung microsomes, EROD activity was analyzed as an enzymatic marker for CYP1A1 induction and biomarker of exposure to the PAH component of ETS. Lung microsomes of ETS-exposed rats showed higher EROD activity than those of sham-exposed rats while liver EROD activity was not significantly different between treatment groups

Table 3.1 Environmental tobacco smoke exposure (ETS) conditions, plasma cotinine concentrations, and ethoxyresorufin-o-deethylase activity in lung and liver tissue. Temperature and carbon monoxide (CO) were measured every 30 seconds for a total of one hour (sham) or 20 minutes (ETS) in preliminary experiments in the absence of rats and are expressed as mean CO for a one hour exposure. Particulates were sampled continuously for one hour (sham) or 20 minutes (ETS) and are expressed as total particulate matter for a one hour exposure period. Rats were then exposed daily for one hour to sham (unlit cigarette) or ETS (3 cigarettes) for 28 days. Blood samples collected after exposure on day 28 were used for plasma cotinine quantitation (n=8 rats per group) and data are expressed as mean ± standard error of the mean (SEM). Ethoxyresorufin-o-deethylase (EROD) activity was analyzed in liver and lung microsomes prepared from rats (n=8 per group) after 28 days of ETS or sham exposure. *p<0.01 and **p<0.0001 versus sham-exposed group in unpaired *t*-test.

	Sham	**ETS**
Temperature (ºC)	25	26
Carbon Monoxide (mean ppm)	0	358
Particulates ($\mu g/m^3$)	30	360
Plasma Cotinine (ng/ml)	2.1 ± 0.4	51.6 ± 4.8**
Liver EROD (fmol min^{-1} mg $protein^{-1}$)	0.46 ± 0.03	0.46 ± 0.04
Lung EROD (fmol min^{-1} mg $protein^{-1}$)	0.06 ± 0.02	0.22 ± 0.04*

(Table 3.1). There were no significant differences in body, lung or liver weight of rats exposed to ETS compared to sham after 28 days of exposure (Table 3.2).

To obtain the circadian pattern of blood pressure, data was collected once a week for a period of 24 consecutive hours and was expressed as a change from baseline (exposure minus the values obtained prior to rats entering this experiment). Envirnomental tobacco smoke altered the circadian pattern of systolic pressure, diastolic pressure, heart rate and dP/dt (Figure 3.1) as well as pulse pressure (data not shown). Systolic and diastolic pressures, as well as dP/dt were higher in ETS- compared to sham-exposed rats (Figure 3.1). Blood pressure and dP/dt of sham-exposed rats were slightly lower at week 4 than they were at baseline (a negative change from baseline; Table 3.3) while in the ETS group, blood pressure and dP/dt had increased from the baseline values (a positive change from baseline; Table 3.3). The 24-hour circadian pattern shows that ETS increased systolic and diastolic pressure to a greater extent during the period of light, a time of inactivity/sleep in rats (Figure 3.1). More specifically, there was a significant reduction in the percent dip of systolic and diastolic pressures from the dark/active period to the light/inactive period in the ETS-exposed rats (Table 3.3). When the data was averaged for the entire 24 hour period, the change in systolic and diastolic pressures from baseline were also significantly increased in ETS-exposed rat compared to sham-exposed rats (Table 3.3). In the ETS-exposed group, the 24 hour average blood pressure and heart rate at week 4 of the exposure were 124 ± 1 mmHg (systolic blood pressure), 87 ± 2 mmHg (diastolic blood pressure), 309 ± 5 bpm (heart rate). At 4 weeks of sham exposure, blood pressure and heart rate were 122 ± 1 mmHg (systolic), 84 ± 2 mmHg (diastolic), and 324 ± 10 bpm.

Table 3.2 Body, liver and lung weight of rats exposed daily for one hour to sham (unlit cigarette; n=8 rats) or environmental tobacco smoke (ETS, 3 cigarettes; n=8) for 28 days. Data are expressed as mean ± standard error of the mean (SEM). There were no significant differences between treatment groups in upaired *t*-test (body weight) or analysis of covariance (organ weights) with body weight as a covariate.

	Body Weight (g)	**Liver Weight (g)**	**Lung Weight (g)**
Sham	447 ± 12	18.2 ± 0.7	1.4 ± 0.1
ETS	426 ± 8	17.0 ± 0.8	1.4 ± 0.1

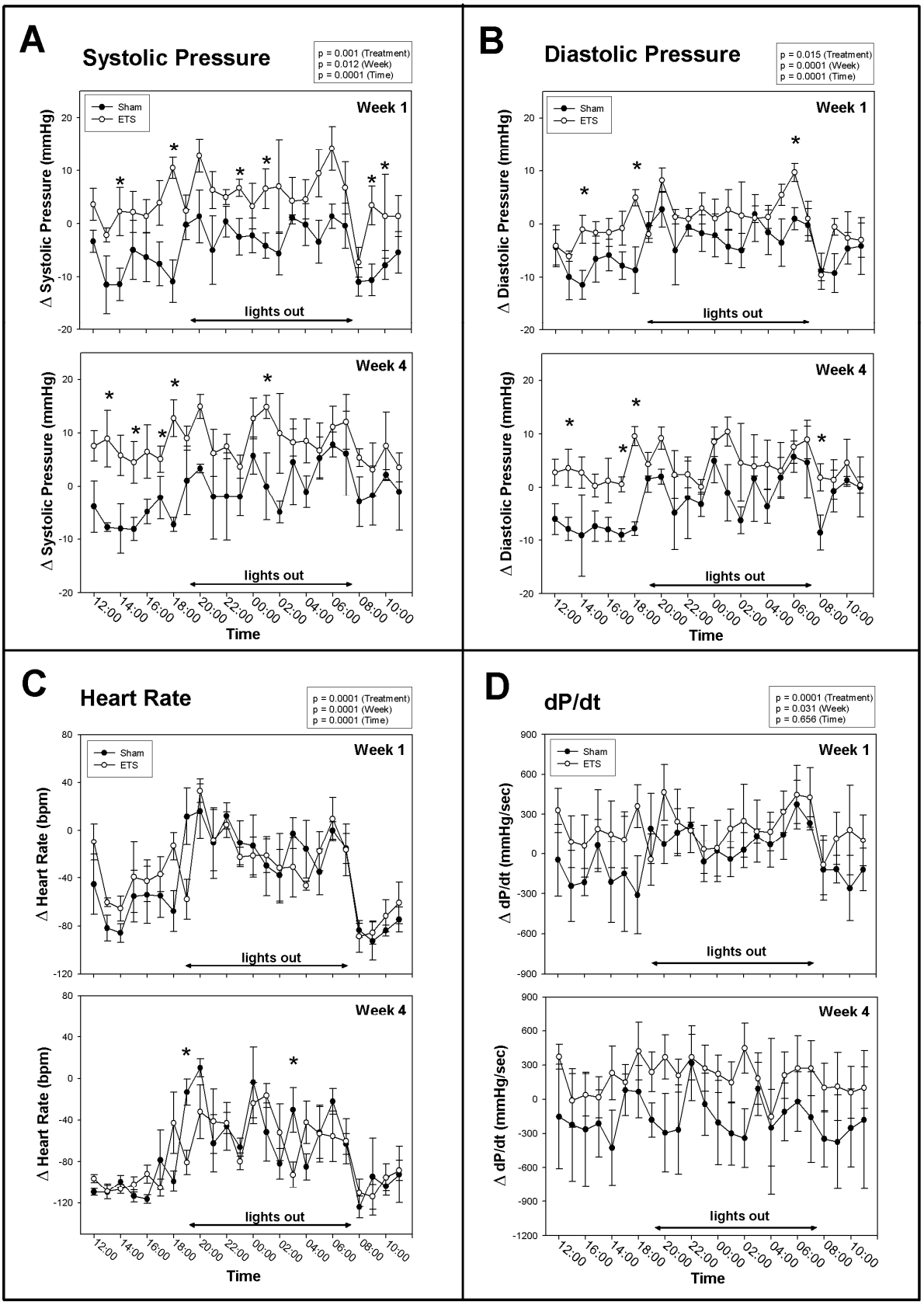

A
Systolic Pressure
p = 0.001 (Treatment)
p = 0.012 (Week)
p = 0.0001 (Time)
Sham
ETS
Week 1
Week 4
Δ Systolic Pressure (mmHg)
lights out
Time
B
Diastolic Pressure
p = 0.015 (Treatment)
p = 0.0001 (Week)
p = 0.0001 (Time)
Δ Diastolic Pressure (mmHg)
C
Heart Rate
p = 0.0001 (Treatment)
p = 0.0001 (Week)
p = 0.0001 (Time)
Δ Heart Rate (bpm)
D
dP/dt
p = 0.0001 (Treatment)
p = 0.031 (Week)
p = 0.656 (Time)
Δ dP/dt (mmHg/sec)

Figure 3.1 Subchronic environmental tobacco smoke (ETS) exposure alters the circadian pattern of A: systolic pressure, B: diastolic pressure, C: heart rate (beats per minute or bpm), and D: dP/dt of the arterial pressure pulse wave. Rats were exposed daily for one hour to sham (unlit cigarette; n=4; filled circles) or ETS (3 cigarettes; n=4; open circles) for 28 days. Results from week 1 and week 4 are shown. Data is expressed as a change in blood pressure, heart rate, or dP/dt from individual pre-exposure values and plotted as mean ± standard error of the mean (SEM). Results were analyzed by 3-way analysis of variance (ANOVA) with treatment, week, and time of day as factors. *$p<0.05$ versus sham-exposed group in Tukey's posteriori test after 3-way ANOVA. There were no significant interactions between the factors in 3-way ANOVA analyses.

Table 3.3 Percent dip and average blood pressure, heart rate (beats per minute or bpm), and dP/dt of the arterial pressure pulse 4 weeks after environmental tobacco smoke (ETS) exposure. Blood pressure telemetry-implanted rats were exposed daily for one hour to sham (unlit cigarette; n=4 rats) or ETS (3 cigarettes; n=4) for 28 days. Blood pressure data collected during a 24 hour period were divided into light and dark periods and expressed as a percent dip (from dark/active to light/inactive period) or were averaged for the entire 24 hours and expressed as a change from individual baseline values. Data are shown as mean ± standard error of the mean. *p<0.05 versus sham-exposed group in unpaired *t*-test.

% Dip at Week 4				
Treatment	**% Systolic Pressure**	**% Diastolic Pressure**	**% Heart Rate**	**% dP/dt**
Sham	6.5 ± 1.0	8.3 ± 2.1	17.1 ± 0.7	7.9 ± 2.2
ETS	-0.3 ± 1.6*	-0.4 ± 1.5*	13.2 ± 1.9	6.6 ± 3.3
24 Hour Average at Week 4				
Treatment	**Δ Systolic Pressure (mmHg)**	**Δ Diastolic Pressure (mmHg)**	**Δ Heart Rate (bpm)**	**Δ dP/dt (mmHg/sec)**
Sham	-2 ± 2	-3 ± 2	-75 ± 2	-457 ± 15
ETS	8 ± 2*	4 ± 1*	-71 ± 11	171 ± 216

In order to better characterize the underlying chronic effects that may be developing, blood pressure data was recorded immediately before the daily exposure. This time was approximately 22 hours after the previous day's exposure and thus the acute effects of the previous day's ETS exposure would be negligible. ETS significantly increased dP/dt by the end of week 1 in this period immediately before daily exposure, and this effect persisted throughout the remaining experiment (Figure 3.2). No significant differences in systolic pressure, diastolic pressure or heart rate were observed at these same times immediately prior to daily exposure (data not shown). Plasma nitrate/nitrite and ET-1 levels were not significantly different between the ETS and sham-exposed rats (Figure 3.2 and Figure 3.3). However, plasma nitrotyrosine levels were significantly increased by ETS exposure at day 28 (Figure 3.3).

Histological analysis of lung tissue showed an increased number of neutrophils in the lungs of rats exposed to ETS after 28 days (Figure 3.4). No other differences in blood cells or pathological changes were noted in the lungs. ETS had no significant effect on heart weight or morphology (Table 3.4) or on left ventricular wall thickness (Figure 3.5). Medial thickness and luminal diameter were measured in histological cross sections of the thoracic aorta, abdominal aorta, and two locations of the iliac branch. There was no significant effect on medial thickness or on the ratio of medial thickness to luminal diameter with 28-day ETS exposure (Table 3.5, Figure 3.5). Furthermore, there were no pathological changes (e.g. no splitting or reduplication of the elastic laminae or vasculitis) evident in arteries from either treatment group after 28 days of exposure.

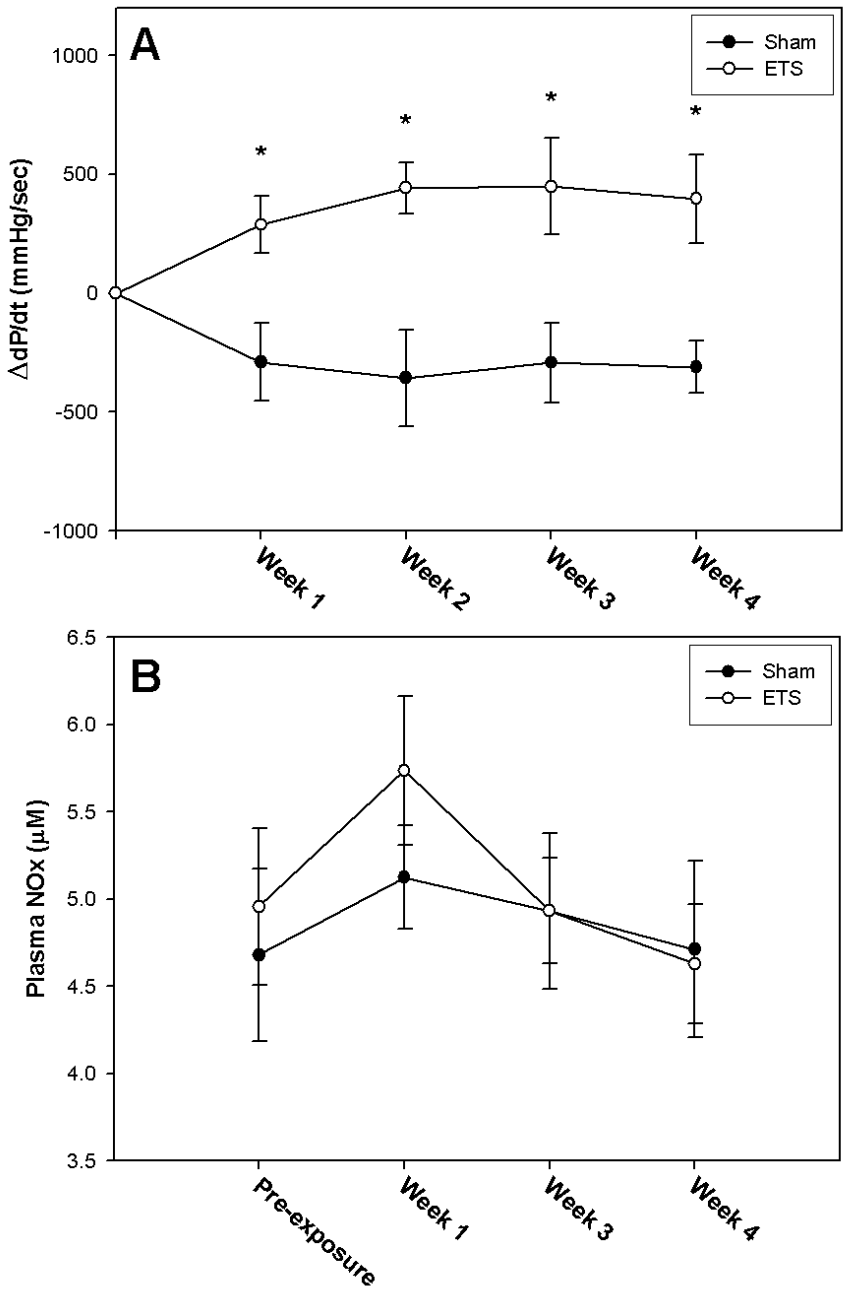
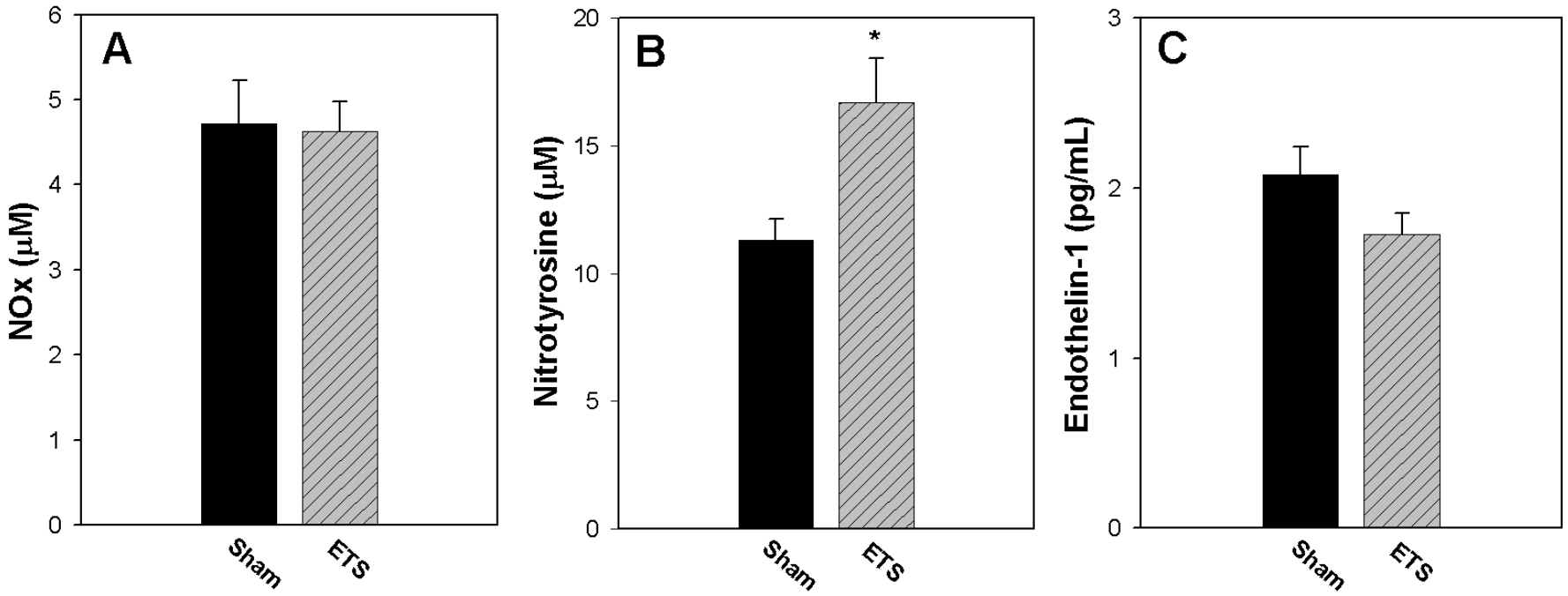


Figure 3.2 A: Pulse wave dP/dt 22 hours after the previous day's environmental tobacco smoke (ETS) exposure and B: plasma nitrate/nitrite (NOx) levels in rats exposed daily for one hour to sham (unlit cigarette; filled circles) or ETS (3 cigarettes; open circles) for 28 days. Resting dP/dt data (n=4 rats per treatment group) was collected over a 20 minute period before daily exposures and blood samples (n=8 per treatment group) were collected after exposure. Data is expressed as a change in dP/dt from pre-exposure values and plotted as mean ± standard error of the mean. *$p<0.05$ versus sham-exposed group in Tukey's posteriori test after 2-way analysis of variance.

Figure 3.3 A: Plasma nitrate/nitrite (NOx), B: nitrotyrosine, and C: endothelin-1 (ET-1) levels in rats exposed daily for one hour to sham (unlit cigarette; n=8 rats; filled bars) or environmental tobacco smoke (ETS; 3 cigarettes; n=8; hatched bars) after 28 days. Data are expressed as mean ± standard error of the mean. *p<0.05 versus sham-exposed group in unpaired *t*-test.



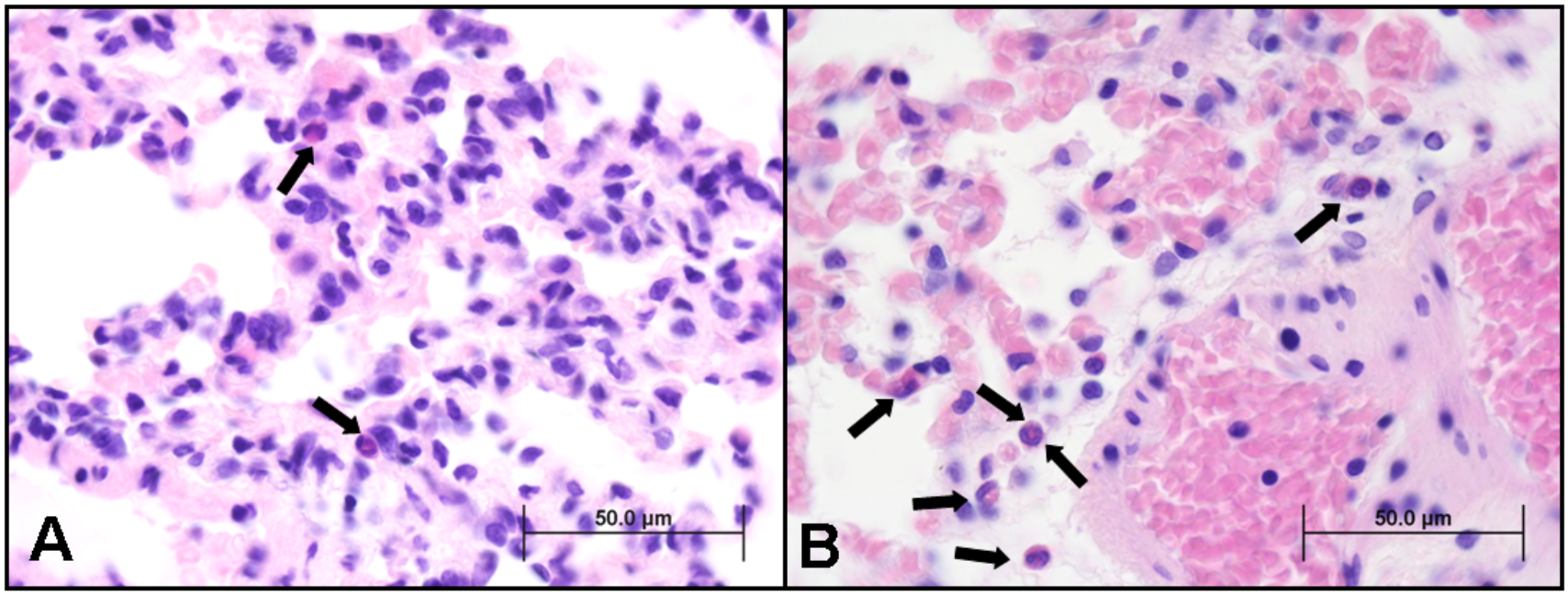


Figure 3.4 Representative photomicrograph showing increased neutrophils (arrows) in the lungs of rats exposed daily for one hour to A: sham (unlit cigarettes) compared to B: environmental tobacco smoke (ETS) (3 cigarettes) after 28 days. Sections were stained with hematoxylin and eosin. Mean number of neutrophils counted at 600x magnification per field of view was significantly increased in ETS-exposed lungs, with sham-exposed rats having 3.0 ± 0.2 neutrophils (mean ± standard error of the mean; n=8 rats) and ETS-exposed rats having 5.4 ± 0.7 neutrophils (n=8; $p<0.05$ compared to sham-exposed group in unpaired *t*-test).

Table 3.4 Heart weight, diameter, and ventricular wall thickness in histological cross sections prepared from rats exposed daily for one hour to sham (unlit cigarette; n=8 rats) or environmental tobacco smoke (ETS, 3 cigarettes; n=8) after 28 days. Data are expressed as mean ± standard error of the mean. There were no significant differences between treatment groups after 1-way analysis of covariance with body weight as a covariate.

	Heart Weight (g)	Whole Heart Diameter (cm)	Ventricular Wall Thickness (mm)
Sham	1.29 ± 0.04	1.4 ± 0.2	1.07 ± 0.04
ETS	1.23 ± 0.04	1.3 ± 0.2	1.09 ± 0.03

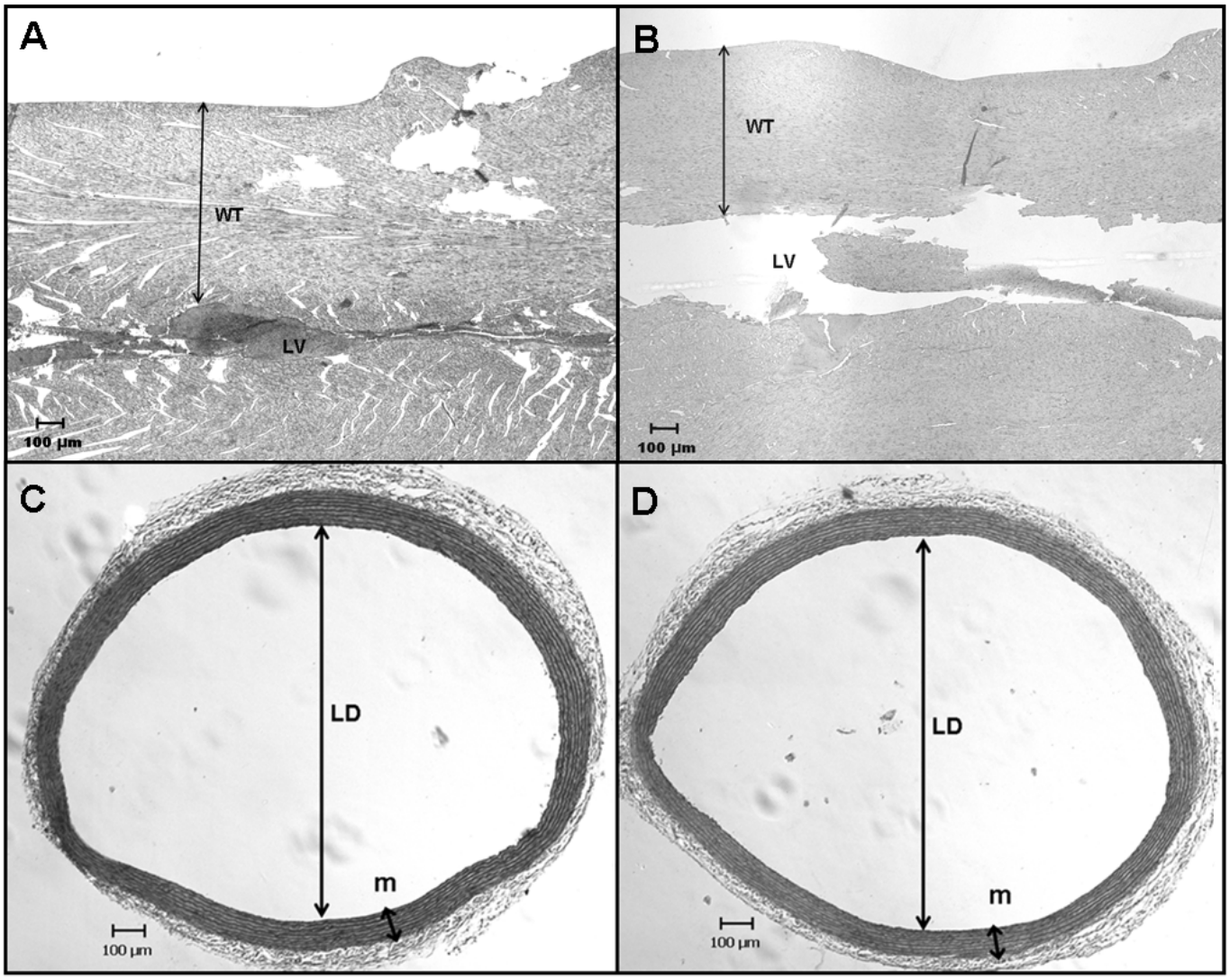


Figure 3.5 Representative photomicrograph of rat heart (A and B) and thoracic aorta (C and D) stained with hematoxylin and eosin showing left ventricular wall thickness (WT), aorta medial thickness (m), and aorta luminal diameter (LD). Sections are from rats exposed daily for one hour to sham (A and C, unlit cigarette) or environmental tobacco smoke (B and D, 3 cigarettes) after 28 days. LV – left ventricular chamber.

Table 3.5 Medial thickness (MT) and ratio of medial thickness to luminal diameter (LD) in histological cross sections of medium and large-sized arteries from rats exposed daily for one hour to sham (unlit cigarette; n=8 rats) or ETS (3 cigarettes; n=8) after 28 days. Data are expressed as mean ± standard error of the mean. No significant differences were found between treatment groups when analyzed by unpaired *t*-test (MT/LD) or by analysis of covariance with body weight as a covariate (MT).

	Thoracic Aorta Medial Thickness (mm)	**Thoracic Aorta MT/LD**	**Iliac Artery Medial Thickness (mm)**	**Iliac Artery MT/LD**
Sham	8.86 ± 0.07	0.080 ± 0.002	7.20 ± 0.07	0.107 ± 0.005
ETS	9.01 ± 0.08	0.076 ± 0.006	7.07 ± 0.02	0.099 ± 0.003

3.4 Discussion

One of the major findings of the current study is that ETS exposure alters the circadian pattern of blood pressure and heart rate, significantly reducing the dipping pattern of blood pressure during sleep. The increase in resting pulse wave dP/dt observed with ETS occurred early in the exposure period and as independent of any changes in blood pressure and heart rate. Furthermore, the increase in pulse wave dP/dt occurred in the absence of any structural alterations of the arterial wall, while NO bioactivity was reduced, suggesting that ETS disrupts functional regulation of arterial mechanical properties before atherosclerosis is clinically manifest in the rat.

Early studies have reported a lower office or clinic blood pressure in smokers compared to nonsmokers (Green et al., 1986; Goldbourt and Medalie, 1977; Savdie et al., 1984; Benowitz, 1989). The chronic effect of cigarette smoke on blood pressure in experimental animals also varies, with reports of unchanged (Haag et al., 1960; Barron et al., 1988; Tanaka et al., 2004) or increased blood pressure in rats (Loscutoff et al., 1982) and mice (Guo et al., 2006). Blood pressure in mammals is known to exhibit a circadian rhythm where blood pressure dips during the night and rises in the morning hours (Millar-Craig et al., 1978), a cycle which is inverted in nocturnal animals (Li et al., 1999). We have observed an average 5% and 8% dip in systolic and diastolic pressures, respectively, in untreated, healthy Sprague Dawley rats using radiotelemetry (see baseline values in Table 4.2). In humans, nondipping (<10% decrease in nocturnal blood pressure) and reverse-dipping (increased nocturnal blood pressure) blood pressure patterns are associated with increased cardiovascular mortality (Pickering and Kario, 2001). Studies in humans have reported that

heart rate (Mikkelsen et al., 1997; Felber Dietrich et al., 2007) and diurnal blood pressure (Mann et al., 1991; Verdecchia et al., 1995; Bolinder and de Faire, 1998; Morillo et al., 2006) are increased in smokers, but nocturnal blood pressure dipping is similar to that of nonsmokers (Morillo et al., 2006). The elevated heart rate and blood pressure during the day are likely due to the acute effects of nicotine from continuous smoking, with withdrawal occurring at night. In contrast, ETS-exposed rats in the current study exhibited non-dipping or reverse dipping blood pressure during sleep. The ETS-induced increases in blood pressure were greatest during the 7 hour period of sleep/inactivity, which immediately followed the daily exposures. Thus, it could be argued that the increase in blood pressure during this sleep period may have been due to the acute effects of ETS. However, previous work from this laboratory indicates that acute ETS exposure in rats (at the same or higher dose) has no effect on or even decreases blood pressure, while increasing heart rate and having no effect on arterial stiffness (see Chapter 2). Thus, we conclude that the observed increase in blood pressure in the current study is not simply due to the acute effects of ETS.

The mechanisms responsible for a loss of nocturnal blood pressure dipping are not completely clear, but there is evidence that endothelial dysfunction may play a role. Nitric oxide has been implicated in the control of circadian variation in blood pressure in rats (Witte et al., 1995) and increased production of ROS can disrupt the timing of circadian clock (Hardeland et al., 2003). In hypertensive individuals, a reversed or nondipping nocturnal blood pressure pattern is associated with increased arterial stiffness (Asar et al., 1996; Lekakis et al., 2005; Jerrard-Dunne et al., 2007). In the current study, ETS significantly altered the 24-hour pattern of pulse wave dP/dt, where the dP/dt of ETS-exposed rats was increased from baseline, indicating increased arterial stiffness.

Changes in arterial stiffness as a result of alterations in smooth muscle tone are considered active changes in arterial elastic properties (Stefanadis et al., 1998), while those due to alterations in transmural distending pressure (Nye, 1964; Stefanadis et al., 1998) and/or heart rate (Bergel, 1961; Mangoni et al., 1996) are considered to be passive. In a previous study, we have demonstrated that pulse wave dP/dt is a useful indicator of active changes in arterial stiffness as a result of acute alterations in smooth muscle tone (see Chapter 2). Chronic smoking or ETS causes structural modifications of the arterial wall (Rahman and Laher, 2007), which are associated with detectable increases in arterial stiffness in humans (Mack et al., 2003). However, there is also evidence that chronic increases in arterial stiffness occur independent of significant structural changes (Safar et al., 2000; Liang et al., 2001 Kameyama et al., 2005). A number of studies have reported that chronic smoking or ETS causes deleterious effects on arterial wall mechanics in healthy, young subjects free of other CVD risk factors (Levent et al., 2004; Li et al., 2005; Kallio et al., 2009). Furthermore, acute (Mahmud and Feely, 2003) and chronic (Liang et al., 2001; Li et al., 2005) increases in arterial stiffness following cigarette smoke occur independent of blood pressure changes. Thus, cigarette smoke appears to have a direct adverse effect on arterial mechanical properties. In the current study, pulse wave dP/dt measured during the time 22 hours after the previous day's ETS exposure was elevated in the ETS-exposed group. This elevated dP/dt occurred in the absence of any changes in blood pressure, heart rate or structural changes in the arterial wall. Thus, the increase in dP/dt appears to reflect active changes in arterial mechanical properties as a result of altered smooth muscle tone.

Previous work from this laboratory indicates that NO influences arterial stiffness, as indicated by pulse wave dP/dt, and that these changes occur independent of blood pressure

changes (see Chapter 2). This is in agreement with earlier studies in humans (Bank et al., 1999; McVeigh et al., 2001; Kinlay et al., 2001; Wilkinson et al., 2002a) and animals (Fitch et al., 2001; Wilkinson et al., 2002b). A reduction in NO bioactivity is a key feature of endothelial dysfunction, and chronic smoking in humans is associated with impaired endothelium-dependent vasodilation in arteries exhibiting increased stiffness (Rehill et al., 2006). In the current study, although plasma ET-1 and NO levels were not significantly altered in ETS-exposed rats, there was an increase in peroxynitrite formation indicating reduced NO bioactivity. Thus, the increase in arterial stiffness in response to ETS exposure in the current study may be due to increased vascular tone as a result of impaired endothelium-dependent vasodilation.

Superoxide anion production within the vasculature represents a mechanism by which NO bioactivity may be reduced (Beckman et al., 1996), and a there are a number of potential sources of free radicals in cigarette smoke. While the gas-phase radicals in cigarette smoke are only stable for a short time, tar-phase radicals can generate additional ROS and are much more stable, having the potential to leave the pulmonary circulation and reach the peripheral vasculature (Pryor et al., 1993, 1998; Smith and Fischer, 2001). For example, PAHs cause AhR-induced increases in the expression of genes such as CYP1A1 and are also metabolically activated by CYP1A1 to form quinone structures that generate large amounts of superoxide anions (Miller and Ramos, 2001). A large number of chemicals in cigarette smoke are cytochrome P450 substrates or inducers (Czekaj et al., 2005) and cigarette smoke has been shown to induce liver CYP1A1 gene expression (Kawamoto et al., 1993; Wardlaw et al., 1998; Czekaj et al., 2005; Rahman et al., 2007). In the current study, the majority of cytochrome P450 substrates were likely metabolized in the lung and peripheral vasculature,

since CYP1A1 activity was increased lung but not liver tissue. Thus, CYP1A1 activity and reactive metabolites may have contributed, at least in part, to the increase in vascular oxidative stress observed in the current study. Recent evidence suggests that cross-talk exists between the AhR signaling pathway and the regulation of circadian rhythms (Shimba and Watabe, 2009). Thus, ETS-induced alteration in circadian blood pressure rhythms may be related to activation of the AhR and its interaction with other clock signaling molecules.

A previous study from this laboratory reported that *ex vivo* exposure of isolated rat tail arteries to the PAH dimethylbenz[*a*]anthracene actually impairs arterial contractility (Weber, 2004) which seems to contradict the finding of increased blood pressure in the current study. One possible unifying explanation for the previous and current observations is that pulmonary exposure to ETS or PAHs produces a different response than exposure of isolated arteries. The induction of CYP1A1 activity with subsequent formation of reactive metabolites in the lung appears to be required to produce the ETS-mediated effects on vascular function. However, in addition to PAHs, other cigarette smoke components such as nicotine (Yugar-Toledo et al., 2005; Rahman and Laher, 2007; Adamopoulos et al., 2009) and acrolein (Jaimes et al., 2004; Yousefipour et al., 2005) have been reported to increase oxidative stress and therefore may also contribute to impairment of endothelial function and/or arterial stiffness.

On the other hand, there is increasing evidence that inflammatory reactions are involved in the pathophysiology of hypertension (Savoia and Schiffrin, 2006) and abnormalities in vascular function, including endothelial dysfunction and arterial stiffness (Gkaliagkousi and Douma, 2009). While the mechanisms are unclear, oxidative stress may be a key player. Cigarette smoke exposure results in the attraction and activation of

macrophages and neutrophils in the lung (Mordelet-Dambrine et al., 1991; Smith and Fischer, 2001; van der Vaart et al., 2004; Czekaj et al., 2002) and circulation (Winkel and Statland, 1981; Abboud et al., 1986; McKarns et al., 1995). Not only can these cells generate ROS themselves (Rennard and Daughton, 1993; Raupach et al., 2006), but they also release proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (Bliss et al., 1999) that can travel via the blood and induce superoxide anion production in vascular smooth muscle cells (De Keulenaer et al., 1998). Indeed, a number of inflammatory markers and cytokines are elevated in smokers (Tracy et al., 1997; Mazzone et al., 2001; Bermudez et al., 2002; Rahman and Laher, 2007) and in animals exposed to ETS (Zhang et al., 2001; Churg et al., 2002). In the current study, there were increased numbers of neutrophils in the lungs of rats exposed to ETS. Thus, ETS-induced increases in lung inflammatory reactions may have been triggered by production of reactive metabolites within the lungs. Inflammatory mediators would then be released from the lungs, possibly causing the observed increases in oxidative stress in the vasculature, leading to reduced NO bioactivity and increased arterial stiffness. However, there are several regulators of smooth muscle tone in addition to ET-1 and NO, and their contribution to the ETS-induced increase in arterial stiffness cannot be ruled out. For example, AngII has been reported to be increased following cigarette smoke exposure (Yu et al., 1992) and has been shown to increase oxidative stress (Griendling et al., 1994; Zhang et al., 1999) and arterial stiffness (Levenson et al., 1981; Cabrera et al., 1988). A recent study also reported that in hypertensive patients, a non-dipping pattern of blood pressure (compared to dipping) was associated with an exaggerated inflammatory response, indicated by higher CRP levels (Tsioufis et al., 2008). Thus, the ETS-induced inflammatory

reactions may have also contributed to the disruption of circadian blood pressure observed in the current study.

In summary, subchronic ETS exposure in rats results in a loss or reversal in the normal dipping pattern of blood pressure during sleep and increases arterial stiffness in the absence of any structural modifications to the arterial wall. These finding were associated with increased neutrophil infiltration and CYP1A1 activity in the lung and a reduction in systemic NO bioactivity. Thus, inflammatory reactions, production of reactive metabolites in the lung, and oxidative stress may play a role in mediating these effects. The current study also highlights the importance of ambulatory monitoring of blood pressure and arterial stiffness in experimental animals.

4.0 INTRANASAL BENZO[A]PYRENE ALTERS CIRCADIAN BLOOD PRESSURE PATTERNS AND CAUSES LUNG INFLAMMATION IN RATS

4.1 Introduction

Polycyclic aromatic hydrocarbons are ubiquitous environmental contaminants that form as byproducts of combustion processes and are thus found in cigarette smoke, vehicle exhaust particles, and air pollution (Hoffman and Hoffman, 1997; Miller and Ramos, 2001; Liu et al., 2007). Epidemiological studies have linked cigarette smoking (Jacobs et al., 1999) and long-term exposure to fine particulate matter air pollution (Dockery et al., 1993; Pope et al., 2002, 2004; Auchincloss et al., 2008) to increased cardiovascular morbidity and mortality in humans, although the underlying mechanisms have not been fully determined. PAHs exert a wide range of toxic effects, many of which can be attributed, in part, to activation of the AhR (Nebert et al., 2000; Miller and Ramos, 2001). PAH toxicity may also be mediated by its reactive intermediates that are generated by multiple metabolic transformations (Nebert et al., 2000).

The vascular endothelium is the first layer of blood vessels to contact circulating environmental contaminants and their metabolites after absorption. This cell layer has been shown to be highly sensitive towards injury caused by AhR agonists (Toborek et al., 1995; Oesterling et al., 2008; Kopf et al., 2008). Endothelium-derived NO and ET-1 function as mutual antagonists in the regulation of basal arterial tone and blood pressure, as well as several other processes involved in the progression of atherosclerosis (De Meyer and

Herman, 1997; Schulz et al., 2004). Inactivation of endothelial-derived NO by ROS is a key feature of endothelial dysfunction which is associated with hypertension (Taddei et al., 2000), cigarette smoking (Rahman and Laher, 2007) and air pollution exposure (Nogueira, 2009). Arterial stiffness is emerging as a powerful, independent predictor of cardiovascular risk (Blacher et al., 1999a, 1999b, 2000; Laurent et al., 2001) and several lines of evidence support a role for NO (Fitch et al., 2001; Kinlay et al., 2001; Wilkinson et al., 2002a, 2002b) and ET-1 (McEniery et al., 2003) in the regulation of arterial stiffness. We have previously demonstrated that pulse wave dP/dt collected from blood pressure telemetry-implanted rats can be used as an indicator of active changes in arterial stiffness in rats (see Chapter 2). Although cigarette smoke (Liang et al., 2001; Li et al., 2005; Kallio et al., 2009) and diesel exhaust particles (Lundbäck et al., 2009) have been reported to increase arterial stiffness in humans, the effect of the PAH component of these contaminants in isolation on arterial stiffness has not yet been studied.

Polycyclic aromatic hydrocarbons such as BaP are known to bind to and activate the AhR, leading to increased expression of phase I and II metabolic enzymes such as CYP1A1/1A2 (Nebert et al., 2000; Miller and Ramos, 2001). Induction of CYP1A1 can lead to the production of ROS (Stohs, 1990; Puntarulo and Cederbaum, 1998; Schlezinger and Stegeman, 2001). Since CYP1A1 metabolically transforms PAHs into quinones which undergo redox cycling, large amounts of superoxide anion can be generated with repeated or chronic AhR agonist exposure (Miller and Ramos, 2001). Both epidemiological and experimental studies indicate that exposure to AhR agonists in the environment can lead to cardiovascular toxicity. PAHs (Burstyn et al., 2005; Aboutabl et al., 2009) and other AhR agonists (Heid et al., 2001; Kanzawa et al., 2004; Kang et al., 2006; Korashy and El-Kadi,

2006; Kopf et al., 2008) have been reported to cause cardiotoxic effects in humans and experimental animals. In humans, TCDD exposure is associated with cardiovascular diseases such as hypertension (Kim et al., 2003; Pesatori et al., 2003; Kang et al., 2006). Polychlorinated biphenyls have been shown to cause endothelial dysfunction and there is evidence that this effect is mediated by increased oxidative stress associated with AhR-induced CYP1A1 activity (Slim et al., 1999; Hennig et al., 2002; Ramadass et al., 2003). TCDD also causes endothelial dysfunction and hypertension (Dalton et al., 2001; Kopf et al., 2008) in experimental animals, which is associated with increased superoxide anion formation (Kopf et al., 2008). Several studies in animal models indicate that BaP increases the development of atherosclerosis (Albert et al., 1977; Penn and Snyder, 1988; Hough et al., 1993; Curfs et al., 2004, 2005) and there is evidence that this may be attributable to increased oxidative stress (Wang et al., 2009; Yang et al., 2009). Polycyclic aromatic hydrocarbons are also well known to cause adverse inflammatory effects in the respiratory system (Nel et al., 2001). Benzo[a]pyrene and other AhR agonists increase the expression of proinflammatory cytokines such as interleukin-8 (IL-8) and TNF-α which in turn cause infiltration of macrophages and neutrophils into the lung (Nel et al., 2001; Podechard et al., 2008; Wong et al., 2010). Both circulating and in-situ activated macrophages and neutrophils can generate ROS (Rennard and Daughton, 1993). Furthermore, inflammatory cytokines can induce the production of oxygen free radicals in numerous cell types, including vascular smooth muscle cells (De Keulenaer et al., 1998).

Thus, we hypothesize that inflammatory reactions and the production of PAH reactive metabolites may serve as a source of oxidative stress leading to reduced NO bioactivity in the peripheral vasculature. While a large number of studies have investigated the carcinogenic

and atherogenic effects of PAHs, few previous studies have examined the effects of PAH injection on blood pressure (Kellen and Anderson, 1972; Ichihara et al., 2009), and none after exposure via the airways. Furthermore, there is evidence that cross-talk exists between the AhR signaling pathway and the regulation of circadian rhythms (Shimba and Watabe, 2009). The objective of this study was therefore to determine whether daily intranasal BaP exposure for 7 days in rats would induce changes in arterial stiffness and circadian patterns of blood pressure related to systemic oxidative stress, pulmonary inflammation, and/or increased pulmonary CYP1A1 activity. Both functional (ET-1, NO production and bioactivity) and structural (wall thickness) features of the arterial wall were also examined.

4.2 Materials and Methods

4.2.1 Animals and Surgery

Animals and surgery, liver and lung EROD activity, histological analysis, and measurement of plasma NOx, ET-1, and nitrotyrosine were conducted as described elsewhere (Chapter 2 and 3). Briefly, all protocols were approved by the Animal Care and Use Council at the University of Saskatchewan in accordance with the Canadian Council on Animal Care guidelines. Rats (195-225g) were housed individually under standard conditions with food and water available *ad libitum* except during exposures.

Rat (n=4 per treatment group) were administered midazolam (0.2 mg/ml i.m.; Sandoz Canada, Inc., Boucherville, CAN) and buprenorphine (20 μg/kg i.m.; Schering-Plough, Hertfordshire, UK). Anaesthesia was initiated at 5% isoflurane (Abbott Laboratories, Saint-

Laurent, CAN) and maintained at 3% isoflurane. A PA-C10 radiotelemetry blood pressure transmitter (Data Sciences International, St. Paul, USA) was implanted into the femoral artery and advanced toward the iliac artery with the transmitter body placed subcutaneously in the left flank. Post-operative treatment included thermal support for 1 hr, fluid replacement immediately post-operative (5-10 ml saline s.c.), buprenorphine (20 μg/kg i.m. every 12 hr for up to 48 hr), and trimethoprim/sulfadoxine (30 mg/kg i.m. daily; Schering-Plough, Hertfordshire, UK for 7 days). Rats were allowed to recover from surgery for at least 14 days before experiments.

4.2.2 Benzo[a]pyrene Exposure

Rats (n=6 per treatment group) were anaesthetized with 3% isoflurane and administered an intranasal dose of vehicle (0.2 ml/kg tricaprylin, T9126, Sigma-Aldrich, St. Louis, USA, 99%) or 0.01 mg/kg benzo[a]pyrene (Sigma-Aldrich, St. Louis, USA, 96%) each day for a total of 7 days. Animals were allowed to recover from anaesthesia for at least 3 hours before blood pressure recordings were initiated.

4.2.3 Plasma Nitrate/Nitrite, Endothelin-1, and Nitrotyrosine Quantitation

Blood samples were collected from rats approximately 30 minutes after vehicle and BaP exposures. Plasma nitrate/nitrite levels were measured using a commercially available enzyme-based kit (Nitric Oxide Quantitation Kit, Active Motif North America,

Carlsbad, USA). Plasma ET-1 (R&D Systems, Inc, Minneapolis, USA) and nitrotyrosine (Cell Sciences, Canton, USA) levels were quantitated using commercially available enzyme-linked immunosorbent assays.

4.2.4 Lung and Liver Ethoxyresorufin-o-deethylase Activity

Rat liver and lung were homogenized (PT10135 homogenizer; Brinkmann Instruments Co., Mississauga, Ontario, CAN) in ice-cold 0.05 M Tris (Sigma-Aldrich, St. Louis, USA)/1.15% KCl (EMD Biosciences, San Diego, USA, 99% purity) (pH 7.5) buffer solution. The ratio of tissue to homogenization buffer was 2 g/25 ml. The homogenate was immediately centrifuged at 10,000g at 4 °C for 20 min in an RC5C centrifuge (Sorvall instruments, Mandel Scientific Co. Ltd., Guelph, CAN). Microsomes in the supernatant were then pelleted at 100,000g at 4 °C for 60 min in a Sorvall WX Ultra 80 ultracentrifuge (Thermo Electron Corp., Waltham USA). The pellet was resuspended in 0.25 M sucrose (EMD Biosciences, San Diego, USA, molecular biology grade) and frozen at -80º C, until analysis.

Protein concentrations in liver and lung microsomal preparations were determined using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Mississauga, CAN) using bovine serum albumin (Sigma-Aldrich, St. Louis, USA) as a calibration standard. For the EROD assay, standards were prepared from resorufin sodium salt (Sigma-Aldrich, St. Louis, USA) diluted with dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA). Beta-nicotinamide adenine dinucleotide phosphate (NADPH Sigma-Aldrich, St. Louis, USA, 94%) was dissolved in water (1.25 mM) just before preparation of the sample series. The reaction was initiated by addition of NADPH to the sample solution. Samples were analyzed

in the following assay conditions: pH 7.4 (37°C) for 75 min in a mixture of Hepes buffer (75.5 mM, Sigma-Aldrich, St. Louis, USA, 99.5%) with $MgCl_2$ (3.77 mM, EMD Biosciences, San Diego, USA, molecular biology grade), ethoxyresorufin (5 μM, Sigma-Aldrich, St. Louis, USA, 95%) dissolved in DMSO (5 μM), 20 μg liver or lung protein, and NADPH (0.25 mM) in a final volume of 250 μl. A positive control sample (liver microsomes prepared from rainbow trout injected 2x with 10 mg/kg benzo[a]pyrene over 72 hrs) was analyzed in every sample series to ensure assay performance.

4.2.5 Histological Analysis

Whole heart, a portion of the lung (lower left lobe), thoracic aorta immediately after the arch, and the abdominal aorta with iliac branches were dissected from rats euthanized at day 8 of treatment (n=6 per treatment group) and transferred into neutral buffered formalin. After 24 hours, samples were transferred to 70% ethanol and stored until histological analysis could be performed. Samples were paraffin-embedded and cross-sectioned (5 μm serial sections), then stained with hematoxylin and eosin. For the heart, 3 serial sections were analyzed at 4 mm above the apex. Size analysis of the heart was performed using digital photomicrographs obtained with an Olympus SZ61 dissecting microscope and 5.0 Megapixel Olympus Q-Color5™ Camera System (Olympus Canada Inc., Markham, CAN). Photomicrographs were taken at 11x magnification and analyzed via Image-Pro software. All other analyses utilized digital photomicrographs obtained with a Zeiss AxioVert light microscope. Heart and vascular analysis were photographed at 50x magnification and analyzed using AxioVision software. For the heart, left ventricular wall thickness was measured in 3 serial sections and averaged. For each artery, the average luminal diameter

was averaged from 3 serial sections, being derived from the luminal area: LD = 2*square root(area/π). The outer artery area was similarly used to derive the average outer diameter in 3 sections per rat. Medial thickness was then calculated from these values: MT = (OD-LD)/2. For the lung, cross sections were scanned for 4 zones of high neutrophil numbers, where the number of neutrophils was counted at 400x magnification. A single mean value per rat was used for all statistical comparisons.

4.2.6 Data Evaluation and Statistical Analysis

To obtain circadian blood pressure patterns over the seven day exposure period, blood pressure data was collected daily for a period of 20 consecutive hours (10 minutes of sampling per rat per hour), starting at least 3 hours after the daily exposure and ending prior to the exposure period the next day. In order to minimize effects of variation among individuals in baseline blood pressure values, pre-exposure (baseline or day 0) blood pressure, heart rate, and dP/dt values were subtracted from post-exposure values to determine the treatment effects. All data are expressed as mean ± SEM. Differences among groups were detected using unpaired, two-tailed *t*-test (single time point data), two-way (data with factors for day of exposure and treatment), or three-way (data with factors for time of day, day of exposure and treatment) analysis of variance (ANOVA) followed by Tukey's posteriori tests as appropriate. However, all organ weights and tissue sizes were analyzed by ANCOVA with body weight as a covariate.

4.3 Results

There were no significant differences in body, lung or liver weight of rats exposed to BaP compared to vehicle after 7 days of exposure (Table 4.1). Benzo[a]pyrene altered the circadian pattern of systolic pressure, diastolic pressure, and heart rate (Fig 4.1). Both blood pressure and heart rate were higher in BaP-exposed rats compared to vehicle control. Pulse wave dP/dt was not significantly altered in BaP-exposed rats (Figure 4.1 and Table 4.2). The 20-hour circadian pattern shows that BaP increased systolic and diastolic pressure to a greater extent during the period of light, a time of inactivity/sleep in rats. This was confirmed by a significant reduction in the percent dip of systolic and diastolic pressures from the dark/active period to the light/inactive period in the ETS-exposed rats (Table 4.2). When the data was averaged for the entire 20 hour period, the change in diastolic pressure from baseline was significantly increased compared to vehicle at day 7 of exposure (Table 4.2). In the BaP-exposed group, the 20 hour average blood pressure and heart rate at day 7 of the exposure were 124 ± 2 mmHg (systolic blood pressure; mean ± SEM; n=4), 87 ± 2 mmHg (diastolic blood pressure), 380 ± 8 bpm (heart rate). After 7 days of vehicle exposure, blood pressure and heart rate were 121 ± 3 mmHg (systolic; n=4), 82 ± 3 mmHg (diastolic), and 375 ± 2 bpm (heart rate).

Plasma nitrate/nitrite levels, nitrotyrosine, and ET-1 were not significantly changed in the BaP- compared to vehicle-exposed rats (Figure 4.2). Neither lung nor liver tissues of BaP-exposed rats had significantly increased EROD activity compared to vehicle-exposed rats (Figure 4.3). Histological analysis of lung tissue showed increased number of neutrophils in the lungs of rats exposed to BaP after 7 days (Figure 4.4). No other differences in blood

Table 4.1 Body, liver, and lung weight of rats administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=6 rats) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=6) after 7 days. Data are expressed as mean ± standard error of the mean. There were no significant differences between treatment groups after 1-way analsysis of variance (body weight) or analysis of covariance (organ weights) with body weight as a covariate.

	Body Weight (g)	**Liver Weight (g)**	**Lung Weight (g)**
Vehicle	325 ± 8	15.8 ± 0.5	1.4 ± 0.1
BaP	335 ± 6	16.0 ± 0.5	1.7 ± 0.2

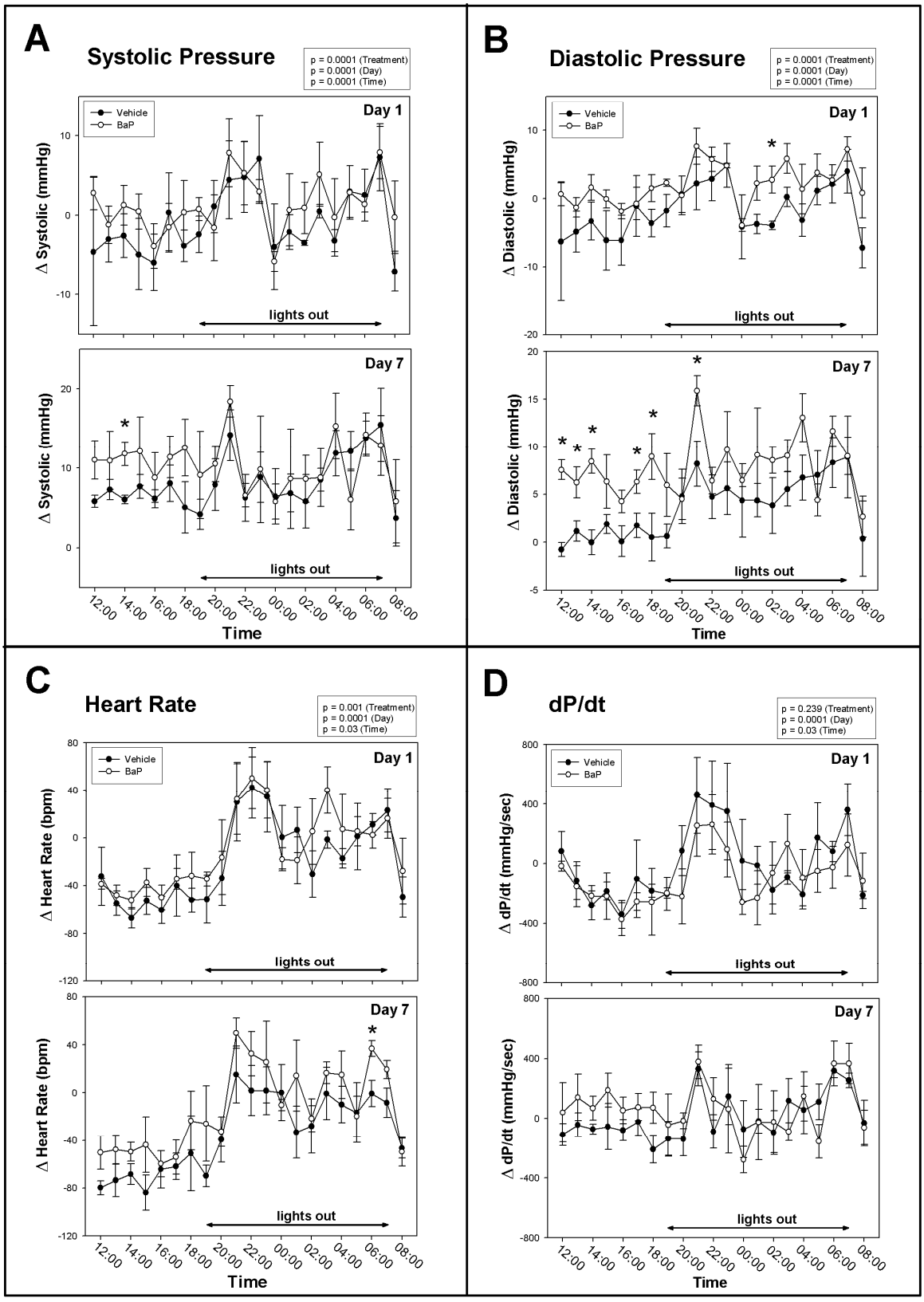

A
Systolic Pressure
p = 0.0001 (Treatment)
p = 0.0001 (Day)
p = 0.0001 (Time)
Vehicle
BaP
Day 1
Δ Systolic (mmHg)
lights out
Day 7
Time
B
Diastolic Pressure
p = 0.0001 (Treatment)
p = 0.0001 (Day)
p = 0.0001 (Time)
Δ Diastolic (mmHg)
C
Heart Rate
p = 0.001 (Treatment)
p = 0.0001 (Day)
p = 0.03 (Time)
Δ Heart Rate (bpm)
D
dP/dt
p = 0.239 (Treatment)
p = 0.0001 (Day)
p = 0.03 (Time)
Δ dP/dt (mmHg/sec)
12:00
14:00
16:00
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00

Figure 4.1 Benzo[a]pyrene (BaP) alters the circadian pattern of A: systolic pressure, B: diastolic pressure, and C: heart rate but has no significant effect on the circadian pattern of D: dP/dt of the arterial pressure wave. Rats were administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=4 rats) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=4) for 7 days. Data is expressed as a change in blood pressure, heart rate, or dP/dt from individual pre-exposure values and plotted as mean ± standard error of the mean. Results were analyzed by 3-way analysis of variance (ANOVA) with treatment, day of exposure, and time of day as factors. *$p<0.05$ versus sham-exposed group in Tukey's posteriori test after 3-way ANOVA. There were no significant interactions between the factors in 3-way ANOVA analyses.

Table 4.2 Percent dip and average blood pressure, heart rate (beats per minute or bpm), and dP/dt of the arterial pressure pulse wave after benzo[a]pyrene (BaP) exposure. Blood pressure telemetry-implanted rats were administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=4 rats) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=4) for 7 days. Blood pressure data collected during a 20 hour period were divided into light and dark periods and expressed as a percent dip (from dark to light period; upper table) prior to exposure experiments (baseline) or at Day 7 of exposure. Alternatively, blood pressure data was averaged for the entire 20 hours and expressed as a change from individual baseline values (lower table). Data are shown as mean ± standard error of the mean. *p<0.05 versus vehicle group in unpaired *t*-test.

% Dip at Baseline and Day 7					
Time/Treatment		**% Systolic Pressure**	**% Diastolic Pressure**	**% Heart Rate**	**% dP/dt**
Baseline		5.0 ± 1.4	7.7 ± 1.8	11.1 ± 0.9	12.7 ± 2.5
Day 7	**Vehicle**	4.0 ± 0.2	5.3 ± 1.1	13.8 ± 2.2	4.4 ± 2.4
Day 7	**BaP**	-0.4 ± 0.5*	2.1 ± 0.4*	13.9 ± 1.1	-2 ± 4.2
20 Hour Average at Day 7					
Time/Treatment		**Δ Systolic Pressure (mmHg)**	**Δ Diastolic Pressure (mmHg)**	**Δ Heart Rate (bpm)**	**Δ dP/dt (mmHg/sec)**
Day 7	**Vehicle**	8 ± 1	3 ± 1	-38 ± 7	31 ± 24
Day 7	**BaP**	10 ± 3	8 ± 1*	-19 ± 11	52 ± 99

Figure 4.2 A: plasma nitrate/nitrite (NOx), B: nitrotyrosine, and C: endothelin-1 (ET-1) levels in rats administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=6 rats; solid bars) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=6; hatched bars) after 7 days. Data are expressed as mean ± standard error of the mean. No significant differences were found between treatment groups in unpaired *t*-test.

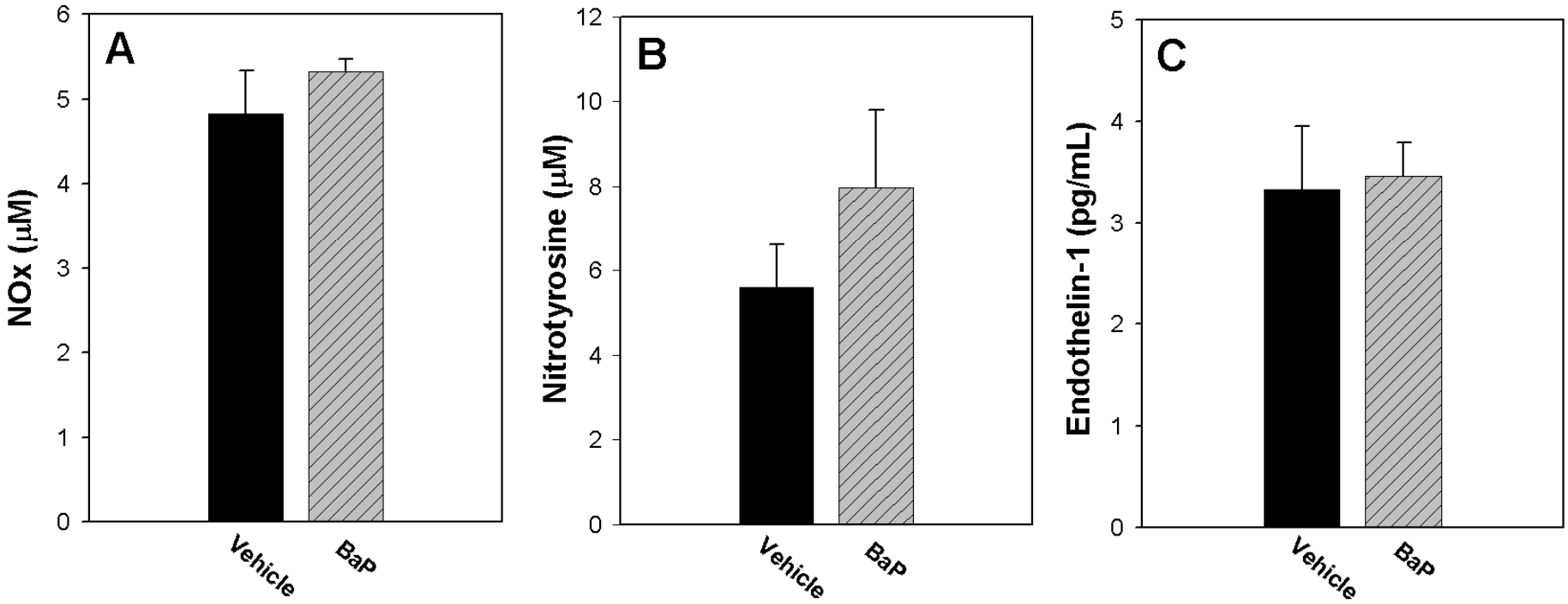


Figure 4.3 Ethoxyresorufin-o-deethylase (EROD) activity in liver and lung microsomes collected from rats administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=6 rats; solid bars) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=6; hatched bars) after 7 days. Data are expressed as mean ± standard error of the mean. No significant differences were found between treatment groups in upaired *t*-test.

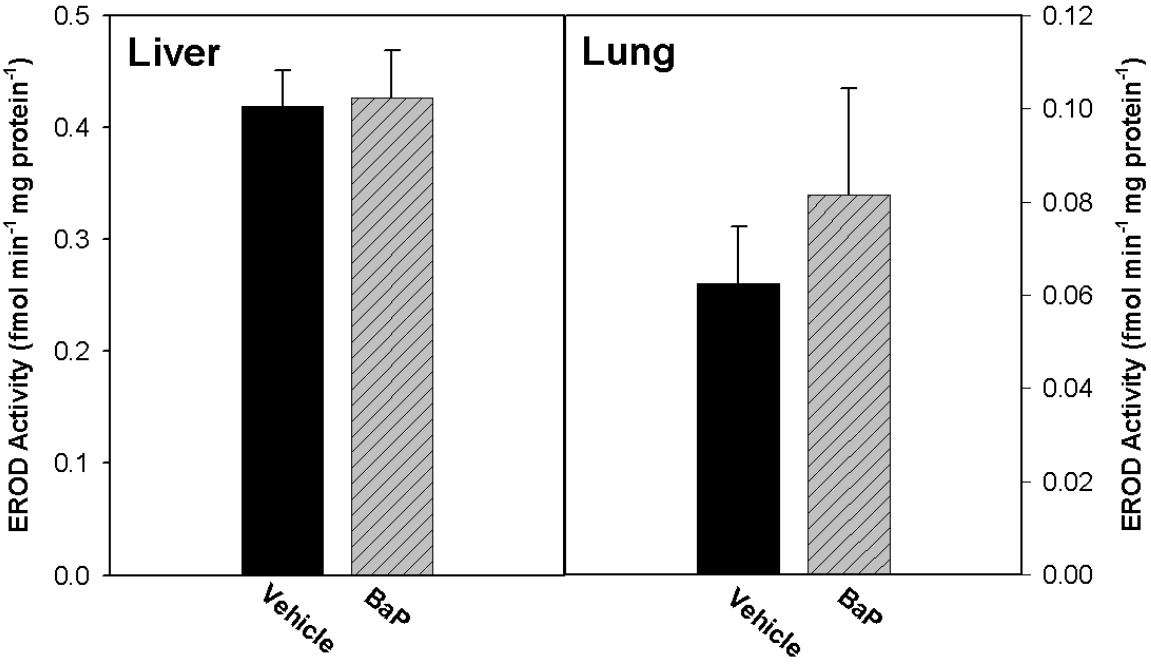
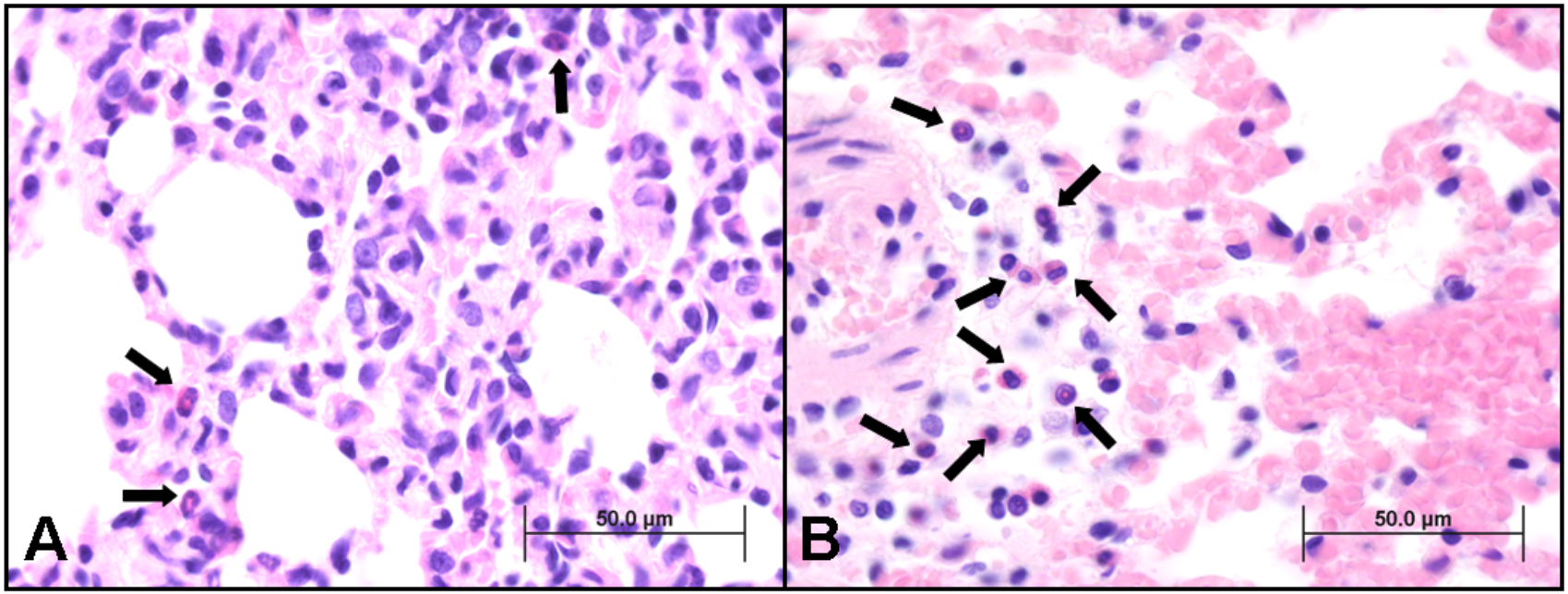


Figure 4.4 Representative photomicrograph showing increased neutrophils (arrows) in the lungs of rats administered a daily intranasal dose of A: vehicle control (tricaprylin, 0.2ml/kg; n=6 rats) compared to B: benzo[a]pyrene (BaP) (0.01 mg/kg; n=6) after 7 days. Sections were stained with hematoxylin and eosin. Mean number of neutrophils counted at 600x magnification per field of view was significantly increased in BaP-exposed lungs, with vehicle-exposed rats having 3 ± 0.5 (vehicle; mean ± standard error of the mean) and BaP-exposed rats having 8 ± 0.6 neutrophils ($p<0.05$ compared to vehicle -exposed group in upaired *t*-test).



cells or pathological changes were noted in the lungs. BaP had no significant effect on heart weight or morphology or on left ventricular wall thickness (Table 4.3 and Figure 4.5). Medial thickness and luminal diameter were measured in histological cross sections of the thoracic aorta, abdominal aorta, and two locations of the iliac branch. There was no significant effect on medial thickness, on the ratio of medial thickness to luminal diameter or arterial histopathology with BaP exposure (Table 4.4, Figure 4.5).

4.4 Discussion

The results of the current study demonstrate that BaP alters the circadian pattern of blood pressure and heart rate, significantly reducing the dipping pattern of blood pressure during sleep. This BaP-induced increase in blood pressure is in agreement with an earlier study in which injection of the synthetic PAH, dimethylbenz[a]anthracene, caused hypertension in rats 3 months after exposure (Kellen and Anderson, 1972).

Blood pressure in mammals is known to exhibit a circadian rhythm where blood pressure dips during sleep (Millar-Craig et al., 1978; Li et al., 1999). In humans, nondipping (<10% decrease in nocturnal blood pressure) and reverse-dipping (increased nocturnal blood pressure) blood pressure patterns are associated with increased cardiovascular mortality (Verdecchia et al., 1994; Pickering and Kario, 2001). The mechanisms responsible for a loss of blood pressure dipping during sleep are not completely clear, but there is evidence that endothelial dysfunction may play a role. Nitric oxide has been implicated in the control of circadian variation in blood pressure in rats (Witte et al., 1995) and increased production of ROS can disrupt the timing of the circadian clock (Hardeland et al., 2003; Zheng et al.,

Table 4.3 Heart weight, diameter, and ventricular wall thickness in histological cross sections prepared from rats administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=6 rats) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=6) after 7 days. Data are expressed as mean ± standard error of the mean. There were no significant differences between treatment groups after 1-way analysis of covariance with body weight as a covariate.

	Heart Weight (g)	Whole Heart Diameter (cm)	Ventricular Wall Thickness (mm)
Vehicle	1.1 ± 0.1	1.3 ± 0.3	1.25 ± 0.05
BaP	1.1 ± 0.1	1.3 ± 0.2	1.21 ± 0.04

Figure 4.5 Representative photomicrograph of rat heart (A and B) and thoracic aorta (C and D) stained with hematoxylin and eosin showing left ventricular wall thickness (WT), aorta medial thickness (m), and aorta luminal diameter (LD). Sections are from rats after 7 days of a daily intranasal dose of vehicle (A and C, tricaprylin, 0.2ml/kg) or benzo[a]pyrene (B and D, 0.01 mg/kg). LV – left ventricular chamber.

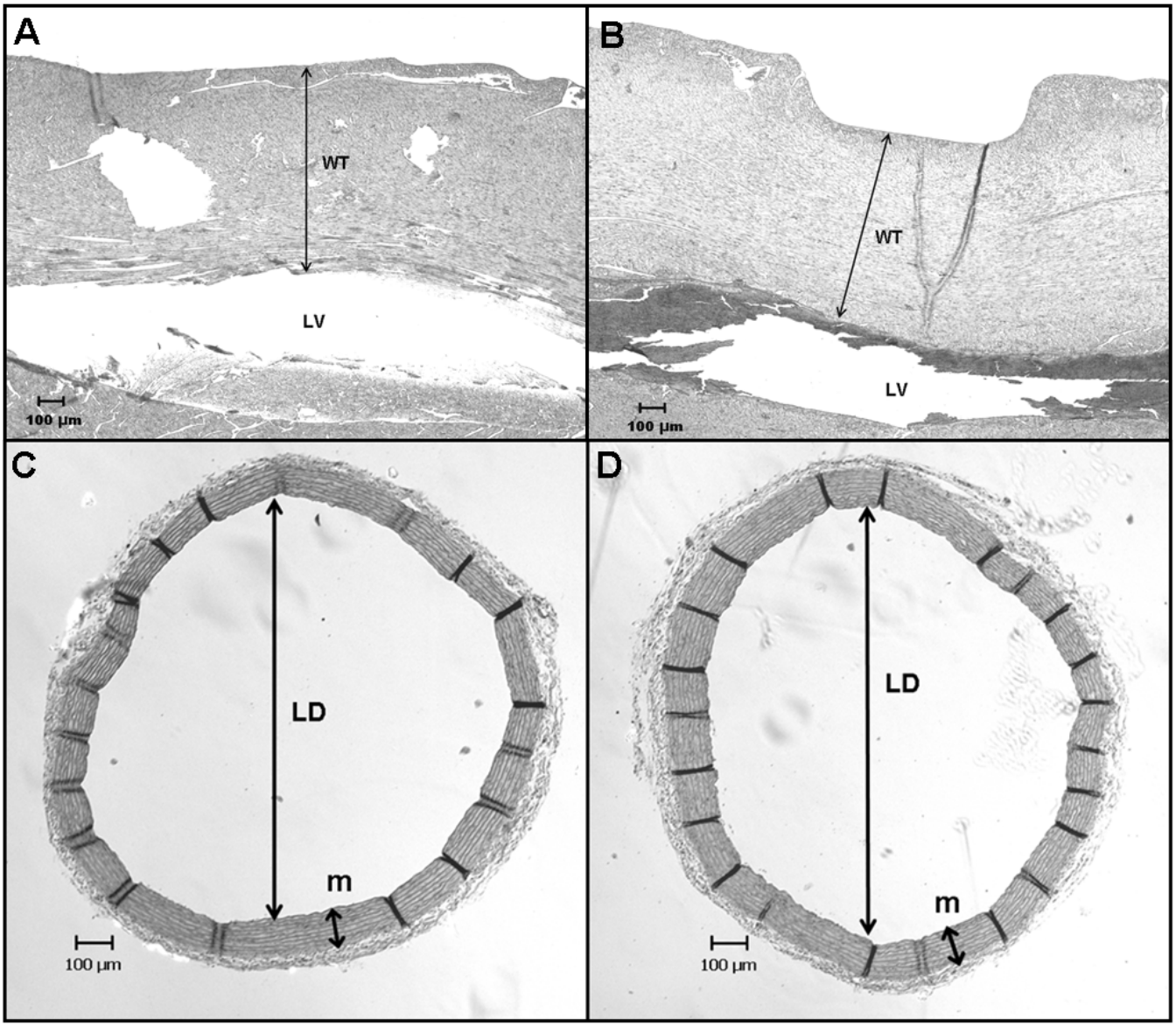


Table 4.4 Medial thickness (MT) and ratio of medial thickness to luminal diameter (LD) in histological cross sections of medium and large-sized arteries from rats administered a daily intranasal dose of vehicle control (tricaprylin, 0.2 ml/kg; n=6 rats) or benzo[a]pyrene (BaP, 0.01 mg/kg; n=6) for 7 days. Data are expressed as mean ± standard error of the mean. No significant effects of treatment were detected in upaired *t*-test (MT/LD) or after 1-way analysis of covariance with body weight as a covariate (MT).

	Thoracic Aorta Medial Thickness (mm)	**Thoracic Aorta MT/LD**	**Iliac Artery Medial Thickness (mm)**	**Iliac Artery MT/LD**
Vehicle	7.29 ± 0.04	0.090 ± 0.004	6.45 ± 0.06	0.109 ± 0.013
BaP	7.94 ± 0.05	0.094 ± 0.005	6.46 ± 0.01	0.099 ± 0.007

2007). In humans, exposure to other AhR agonists such as TCDD is associated with increased incidence of hypertension (Pesatori et al., 2003; Kim et al., 2003; Kang et al., 2006). A previous study reported that TCDD exposure in mice caused hypertension with non-dipping blood pressure patterns during sleep, which was associated with increased superoxide anion production and endothelial dysfunction (Kopf et al., 2008). Therefore this failure of blood pressure to dip may be a common response to AhR agonist exposure, supporting the hypothesis that the activated AhR influences the control of circadian rhythm (Shimba and Watabe, 2009). Consistent with prolonged systemic hypertension, mice exposed to TCDD also developed concentric left ventricular hypertrophy and increased heart weight (Kopf et al., 2008). A recent study also reported that 7-day BaP exposure increased heart weight to body weight ratio with induction of hypertrophic markers (Aboutabl et al., 2009). In contrast, there were no changes in heart weight or left ventricular wall thickness observed in the current study. The reason for the lack of cardiac hypertrophy in the current study is unknown, but likely relates to the shorter exposure time, shorter duration of effect for BaP versus TCDD or to differences in the route of exposure. If either of the former two reasons are explanations, then the results of the current study would suggest that AhR-agonist-induced blood pressure changes precede cardiac changes.

Benzo[a]pyrene is capable of increasing oxidative stress through AhR-mediated increases in CYP1A1 expression and the formation of reactive metabolites (Miller and Ramos, 2001). In the current study, CYP1A1 activity was not significantly induced in lung or liver tissue. Since endothelial cells have been reported to have a greater capacity for CYP1A1 induction in PAH-treated rats compared to hepatic or other tissues (Thirman et al., 1994), BaP that reaches the systemic circulation after absorption from the lung has a high

likelihood of being metabolically activated within the vasculature. However, since NO bioactivity (as indicated by plasma nitrotyrosine) was not significantly altered in the current study, oxidative stress-mediated impairment of endothelial function cannot explain the increase in blood pressure and loss of dipping pattern observed in the current study. Reversed or non-dipping blood pressure patterns have been associated with arterial stiffness in hypertensive patients (Asar et al., 1996; Lekakis et al., 2005; Jerrard-Dunne et al., 2007). However, pulse wave dP/dt was not increased by BaP exposure in the current study. In contrast, a previous study from this laboratory found that 30-day ETS exposure in rats did cause increases in oxidative stress and nitrotyrosine production as well as increased pulse wave dP/dt (see Chapter 3). Therefore, this suggests that there is dissociation between the altered circadian blood pressure pattern and oxidative stress-mediated increases in arterial stiffness caused by inhalation of toxic agents. Although not significantly affected by day 7 of intranasal BaP exposure, if the trends evident in the dP/dt and nitrotyrosine in the current experiment continued, then likely oxidative stress-mediated decreases in NO bioactivity and increased arterial stiffness would have been observed with a longer BaP exposure. In summary, since a 7-day BaP exposure caused a similar failure of blood pressure to dip as the 30-day ETS we observed previously, it suggests firstly that PAHs such as BaP may be important mediators of ETS effects and secondly that oxidative stress is not the major mediator of alterations in circadian blood pressure patterns.

Endothelial dysfunction can be more broadly defined as a disruption in any of the regulatory processes of the endothelium, including an imbalance between vasorelaxing and constricting factors leading to dysregulation of smooth muscle tone (Rubanyi, 1993).

Therefore the BaP-induced increase in blood pressure in the current study may have been mediated by elevated vasoconstricting factors such as Ang II and ET-1. In both humans and animal models, hypertension is often associated with increased circulating Ang II and ET-1 and/or activation of the sympathetic nervous system (Kaplan, 2002). In the current study, however, plasma ET-1 was not significantly altered in BaP-compared to vehicle-exposed rats. Similarly, in TCDD-induced hypertensive mice, plasma ET-1 and Ang II were not significantly altered, although the contribution of ET-1 was not ruled out since plasma ET-1 may not necessarily reflect vasoactive ET-1 (Schiffrin, 2005; Kopf et al., 2008). We have previously reported that *ex vivo* exposure of isolated rat tail arteries to the PAH dimethylbenz[*a*]anthracene actually impairs arterial contractility (Weber, 2004). However, this may indicate that the effects of PAHs on the functional properties of arteries require bioactivation at a site other than the arterial wall and/or increased production of inflammatory mediators in other organs, which then circulate to exert systemic effects.

Intranasal BaP exposure in the current study increased neutrophil recruitment into the lung, consistent with previous reports examining effects of BaP exposure (Nel et al., 2001; Podechard et al., 2008). PAHs have also been reported to cause increased expression of various proinflammatory cytokines such as IL-8 and TNF-α (Lyte and Bick, 1986; Pei et al., 2002; Lecureur et al., 2005; N'Diaye et al., 2006; Knaapen et al., 2007; Podechard et al., 2008). Inflammation appears to play a key role in the development of hypertension (Savoia and Schiffrin, 2006) and alterations in vascular function, including endothelial dysfunction and arterial stiffness (Gkaliagkousi and Douma, 2009). It has been hypothesized that circulating inflammatory mediators could be involved in altering the regulation of vascular tone. In addition to increasing production of oxygen free radicals, cytokines appear to be

important mediators of endothelial dysfunction (Pauletto and Rattazzi, 2006). Although the majority of studies linking inflammation to endothelium dysfunction involve impaired NO bioactivity (Aoki et al., 1989; Hingorani et al., 2000; Fichtlscherer et al., 2000), CRP has been reported to induce ET-1 production in cultured venous endothelial cells (Verma et al., 2002). Thus, while BaP-induced inflammatory reactions have the potential to cause endothelial dysfunction by altering endothelial vasoactive mediators, altered NO or ET-1 levels do not explain the association between lung inflammation and altered circadian pattern of blood pressure in the current study. It was recently reported that a non-dipping pattern of blood pressure was associated with increased CRP levels in hypertensive patients (Tsioufis et al., 2008), suggesting that low grade systemic inflammation may play a role in the disruption of normal circadian rhythms. While the mechanism linking inflammation with altered circadian rhythms of blood pressure is largely unclear, results of the current study suggest that oxidative stress is not involved.

In summary, 7-day intranasal BaP exposure in rats results in a loss or reversal in the normal dipping pattern of blood pressure during sleep, which was associated with an increase in neutrophil recruitment in the lungs. Benzo[a]pyrene did not significantly alter plasma ET-1 or NO bioactivity and arterial stiffness was not increased. Thus the current study provides evidence against our hypothesis that the production of PAH reactive metabolites serve as a source of oxidative stress leading to reduced NO bioactivity in the peripheral vasculature to mediate these alterations in blood pressure. Instead, the current study supports part of our original hypothesis that inflammation, detected only in the lung in this study, is associated with altered circadian rhythm of blood pressure.

5.0 OVERALL DISCUSSION AND CONCLUSIONS

This thesis aimed to determine whether the PAH component of ETS is responsible for the cigarette smoke-induced vascular damage, as well as the pathophysiological mechanisms involved. After confirming that pulse wave dP/dt collected from radiotelemetry-implanted rats can be used an indicator of active changes in arterial stiffness, the cardiovascular effects of environmental tobacco smoke and benzo[a]pyrene were compared.

5.1 Using Pulse Wave dP/dt to Assess Arterial Stiffness in Rats

Arterial stiffness is commonly measured in humans as a surrogate measure of cardiovascular risk. Although various studies in experimental animals have investigated the relationship between structural and functional changes in the arterial wall and measures of arterial stiffness (Katsuda et al., 2000; Wang et al., 2000; Fitch et al., 2001; Tatchum-Talom et al., 2002; Wilkinson et al., 2002b; McEniery et al., 2003; Kameyama et al., 2005), no previous study has examined the effects of ETS exposure on arterial stiffness *in vivo* in experimental animals. Blood pressure radiotelemetry is quickly becoming the gold standard for cardiovascular research as it provides a large amount of reliable, sensitive physiologic data from conscious, unrestrained animals. Experiments in this thesis confirm that the pulse wave dP/dt collected from radiotelemetry-implanted rats can be used as an indicator of active changes of arterial stiffness which occur independent of blood pressure changes. To further use pulse wave dP/dt as an *in vivo* measure of arterial stiffness, it may be important to further characterize acute and chronic changes in pulse wave dP/dt following functional and

structural changes in the arterial wall. For example, future studies should examine the acute effects of other vasoactive mediators, such as ET-1, AngII, and nitroglycerin. Acute inhibition of NO synthesis via L-NAME was demonstrated to cause an immediate, but transiently increased pulse wave dP/dt in this thesis. Reduced NO bioactivity may have also contributed, at least in part, to the increase in arterial stiffness caused by subchronic ETS exposure. Chronic inhibition of NO synthesis by L-NAME leads to early-stage atherosclerosis, including an early inflammatory stage within the first week of treatment and a remodeling stage after 4 weeks (Numaguchi et al., 1995; Takemoto et al., 1997a, 1997b; Tomita et al., 1998). In a previous study in rats, chronic inhibition of NO synthesis via L-NAME treatment resulted in increased pulse wave velocity as early as day 3 of exposure, and preceded the vascular structural changes observed after 1 week of exposure (Kameyama et al., 2005). Since the ETS-induced chronic increase in pulse wave dP/dt in the current study occurred in the absence of structural modifications in the arterial wall, the effects of structural changes in the arterial wall on pulse wave dP/dt are still unknown. Thus, changes in pulse wave dP/dt following chronic reductions in NO alone versus subsequent structural changes in the arterial wall should be examined in future studies. Induction of acute inflammation by vaccination results in endothelial dysfunction and arterial stiffness in humans (Hingorani et al., 2000; Vlachopoulos et al., 2005). Therefore it would also be interesting to determine whether acute or chronic inflammation alone would increase pulse wave dP/dt and blood pressure in rats. Overall, dP/dt extracted from arterial pressure waves provides an *in vivo* measure of active changes in arterial stiffness in rats, and appears to be related to changes in endothelial function.

5.2 Using Radiotelemetry to Examine Circadian Blood Pressure Rhythms in Rats

In future experiments examining circadian blood pressure rhythms in nocturnal animals such as rats, a better exposure model would be to expose them later in the evening or night when they are awake/active rather than immediately before the sleep period. In fact, with the data collection and exposure schedule used in these studies, it would be more accurate to use the term "blood pressure spike" instead of "blood pressure dip", since the time period during which data was collected progressed from the day (inactive period/lower blood pressure) to the night (active period/higher blood pressure).

The ubiquitous nature of clock machinery and its ability to respond to various exogenous stimuli should also be taken into consideration in future studies. For example, circadian rhythms in the expression of the AhR and ARNT as well as downstream targets such as CYP1A1 indicate that the sensitivity of AhR agonists could vary according to the time of treatment (Shimba and Watabe, 2009). There is also significant evidence in humans that rhythms in blood pressure can be uncoupled from endogenous circadian rhythms in activity (Rudic and Fulton, 2009). Thus, performing exposures closer to the periods of activity could minimize potential disruptions to the endogenous behavioural/locomotor/activity circadian rhythms and prevent a loss of synchronization between central and peripheral clocks. The telemetry devices used in this study also record activity in the animal. Therefore, it would be interesting to compare changes in circadian rhythms of blood pressure and heart rate to rhythms in activity (behavior). There is also evidence that energy metabolism can be entrained by the feeding schedule independent of the

SCN (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001; Satoh et al., 2006). Thus, changes in metabolism can lead to an uncoupling of peripheral clocks from the central pacemaker. For example, food restriction in rodents uncouples the peripheral clock in the liver from the central clock (the SCN) (Damiola et al., 2000; Stokkan et al., 2001). As a result, it may also be important to minimize food rewards during the daytime/period of inactivity in rats, particularly since there is a relationship between the circadian clock, metabolism, and obesity known to influence blood pressure (Rudic and Fulton, 2009).

5.3 Acute Versus Subchronic Environmental Tobacco Smoke

There have been few experimental studies investigating multiple effects of cigarette smoke exposure on structural and functional properties of the arterial wall. This thesis simultaneously examined the effects of ETS exposure on structural changes in the arterial wall, *in vivo* measures of arterial stiffness, circadian blood pressure patterns, markers of endothelial function, as well as possible sources of oxidative stress.

In both acute and subchronic ETS exposures in this thesis, plasma nitrotyrosine levels were increased. However ET-1 levels were not significantly altered and plasma NOx was either unchanged or increased following ETS exposure. Peroxynitrite is known to uncouple the eNOS enzyme and inhibit production of NO. However, it has also been demonstrated that following stimulation of cells by proinflammatory cytokines, NO is produced in large quantities by the inducible NO synthase (iNOS) isoform in phagocytes as well as in cells of the vessel wall (Adeagbo and Triggle, 1993; MacMicking et al., 1997; De Meyer and Herman, 1997). Since the induction of iNOS usually occurs in an oxidative environment, this

high concentration of NO is viewed as toxic, since NO reacts with superoxide anion to form peroxynitrite and cause cell toxicity (Moilanen and Vapaatalo, 1995; Mungrue et al., 2002). Peroxynitrite has also been shown to increase iNOS protein levels in endothelial cells via activation of nuclear factor-κB (NF-κB) (Cooke and Davidge, 2002). Thus, under conditions of oxidative stress, increased NO production by iNOS would enhance peroxynitrite formation, resulting in a positive feedback loop and reducing NO bioactivity. Peroxynitrite was also shown to reduce prostacyclin synthase protein levels, which could lead to inhibition of prostacyclin-mediated vasodilation while favoring thromboxane-mediated vasoconstriction, thereby further contributing to vascular dysfunction (Cooke and Davidge, 2002). Thus, depending on the extent of biological inactivation by superoxide anion, both cigarette smoke-induced ROS production and inflammatory reactions could potentially lead to increased NO levels via iNOS upregulation, as was observed with ETS exposures in this thesis. This is consistent with an earlier study in which daily cigarette smoke exposure in rats for 2 months resulted in lung inflammation, induction of iNOS in lung tissue, and a dose-dependent increase in serum nitrite levels (Chang et al., 2001).

While both acute and chronic smoking or ETS exposure increase arterial stiffness in humans, studies in this thesis indicate that chronic, but not acute ETS exposure increases arterial stiffness in rats. However, acute injections of vasoactive drugs and L-NAME revealed that acute changes in pulse wave dP/dt may occur rapidly, but then transiently disappear, likely as a result of a compensatory alteration in heart rate. Thus, in order to better characterize acute changes in blood pressure, heart rate, and dP/dt in any future acute ETS exposures, continuous blood pressure recording during the exposure period may be necessary. Humans obviously smoke while awake which is during the day, and ambulatory

blood pressure monitoring indicates that blood pressure is increased in smokers during the day (Mann et al., 1991; Verdecchia et al., 1995; Bolinder and de Faire, 1998; Morillo et al., 2006), while nocturnal blood pressure dipping is unaltered (Morillo et al., 2006). In contrast, in the current subchronic ETS experiment, rats were exposed during the period of sleep/inactivity and there was a loss of blood pressure dipping during sleep/inactivity. The lack of change or even decrease in blood pressure and pulse wave dP/dt after acute ETS exposure, however, suggests that the subchronic ETS-induced increase in blood pressure during the period of sleep/inactivity in rats is not simply due to the acute effects of ETS.

Overall, subchronic ETS exposure increased resting pulse wave dP/dt independent of blood pressure changes and structural modifications of the arterial wall. Both acute and subchronic ETS exposures were demonstrated to cause reductions in NO bioactivity while ET-1 levels were similar between treatment groups. Thus, endothelial dysfunction, characterized by an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors, may have contributed, to the ETS-induced increase in arterial stiffness. Furthermore, ETS chronically disrupts functional regulation of arterial mechanical properties before atherosclerosis is clinically manifest, further clarifying the relationship between changes in arterial stiffness independent of blood pressure and vascular structure.

5.4 Environmental Tobacco Smoke Versus Benzo[a]pyrene

Despite the similar cardiovascular effects of cigarette smoke and air pollution, the effect of the PAH component in isolation on arterial stiffness and circadian blood pressure patterns had not been previously studied. The major goal of this thesis was to compare the

cardiovascular effects of ETS and BaP exposure, the results of which are summarized in Table 5.1.

Subchronic ETS exposure increased arterial stiffness in rats as early as day 7 of the exposure period, while 7-day intranasal BaP exposure had no significant effect on arterial stiffness. The fact that ETS, but not BaP exposure decreased NO bioactivity lends further support to the hypothesis that endothelial dysfunction contributes to the increase in arterial stiffness caused by ETS. Cytochrome P4501A1 activity was increased in lung tissue of rats exposed to ETS, therefore CYP1A1 activity and reactive metabolites may have also contributed, directly or indirectly, to the increase in vascular oxidative stress. Although BaP is capable of increasing oxidative stress via induction of CYP1A1 and the formation of reactive metabolites, there was no increase in CYP1A1 activity in lung or liver tissue of rats exposed to BaP. This suggests that oxidative stress caused by BaP exposure in the current study may have been insufficient to increase arterial stiffness via reduced NO bioactivity. Therefore, either a longer time for BaP exposure is needed before evidence of increased oxidative stress would be observed or BaP is not the component in cigarette smoke that is responsible for the ETS-induced arterial stiffness. There is certainly a trend in the data in this thesis for increased plasma nitrotyrosine levels, lung CYP1A1 activity, and pulse wave dP/dt by BaP exposure. Thus, it is possible that with longer term exposure, BaP would have also increased oxidative stress and arterial stiffness in rats. Endothelial cells have been reported to have a greater capacity for CYP1A1 induction in PAH-treated rats compared to hepatic or other tissues (Thirman et al., 1994). Therefore to further clarify the location of metabolic activation of PAHs, CYP1A1 induction should be examined in the arterial wall following ETS and/or BaP exposure. Also, using transgenic mice that conditionally over express

Table 5.1 Summary of 28-day environmental tobacco smoke exposure effects compared to those of 7-day intranasal benzo[a]pyrene exposure. Arrows indicate direction of significant change of treated compared to control groups. NS - no significant change compared to control. NOx - nitrate/nitrite, ET-1 - endothelin-1, EROD - ethoxyresorufin-o-deethylase

	Environmental Tobacco Smoke	**Benzo[a]pyrene**
Systolic & Diastolic Pressure	↑	↑
dP/dt	↑	**NS**
Plasma NOx	**NS**	**NS**
Plasma ET-1	**NS**	**NS**
Plasma Nitrotyrosine	↑	**NS**
Lung Neutrophils	↑	↑
Lung EROD Activity	↑	**NS**

CYP1A1 in the absence of toxicant or using AhR-null mice, it would be interesting to examine the effects of different levels of CYP1A1 expression on oxidative stress, blood pressure and arterial stiffness.

Both ETS and BaP exposure increased blood pressure, with a significant reduction in blood pressure dipping during sleep. The circadian clock influences both central and peripheral mechanisms of blood pressure of regulation (reviewed in Rudic and Fulton, 2009). Thus, there may be multiple potential mechanisms by which ETS and BaP altered the circadian rhythm of blood pressure. Recent findings have led to the hypothesis that the AhR plays a role in circadian rhythms (Shimba and Watabe, 2009). Results from a recent study suggest that activation of the AhR may affect the ability of the circadian timekeeping system to adjust to changes in environmental lighting by altering expression of the clock genes (Mukai et al., 2008). Thus, it is possible that the altered circadian blood pressure patterns caused by ETS and BaP may be related to activation of the AhR, its interaction with other clock signaling molecules, and possibly to disruptions in the ability to adjust to alterations in the light/dark cycle.

Physiological and behavioural daily rhythms are generated by the SCN. Variations in mental and physical activity from day to night largely influence circadian blood pressure rhythms (Degaute et al., 1991; Dominguez-Rodriguez et al., 2009) and there are various signals that modulate both activity and blood pressure rhythms (Rudic and Fulton, 2009). Diurnal changes in the autonomic nervous system activity, specifically an increase in sympathetic neural activity, occurs upon awakening and largely influences blood pressure rhythms (Dominguez-Rodriguez et al., 2009). Thus, a shift in autonomic control away from parasympathetic towards sympathetic activation could disrupt circadian rhythms in blood

pressure. Both active and passive smoking activate the sympathetic nervous system (Cryer et al., 1976; Winniford et al., 1986; Hausberg et al., 1997) which stiffens both elastic and muscular arteries (Boutouyrie et al., 1994) and increases blood pressure and heart rate, although the long-term effects on blood pressure are unknown. In humans, the sympathetic neural arousal induced by smoking has been reported to last for 24 hours (Benowitz et al., 1984). A recent study reported that in coke oven workers, BaP affects the autonomic nervous function mainly via down regulation of parasympathetic function, which would similarly alter the balance between parasympathetic and sympathetic tone, favoring activity of the sympathetic nerve (Zhang et al., 2008). This heightened sympathetic tone would increase the heart rate, blood pressure, and cause coronary heart disease (Zhang et al., 2008). This is supported by an earlier study which reported a positive correlation between exposure to BaP in male asphalt workers and fatal ischemic heart disease (Burstyn et al., 2005).

In addition to increasing blood pressure, both BaP and ETS exposure caused increased neutrophil recruitment into the lung, suggesting that lung inflammation may be involved in the disruption of circadian blood pressure rhythms. A recent study reported that hypertensive patients with a non-dipping blood pressure pattern are characterized by a pronounced subclinical inflammatory response (Tsioufis et al., 2008). Another study reported that nocturnal blood pressure non-dipping was associated with elevated levels of molecules related to endothelial dysfunction, such as plasminogen activator inhibitor-1 and soluble intracellular adhesion molecule (von Känel et al., 2004). Although numerous studies indicate that inflammation plays a role in the development of hypertension, arterial stiffness, and endothelial dysfunction, there is little data available concerning the potential pathophysiological connection between these effects (Pauletto and Rattazzi, 2006). Even less

data is available concerning a link between inflammation and altered circadian blood pressure rhythms. The melatoninergic system may play a role in the circadian rhythm of the cardiovascular system (Dominguez-Rodriguez et al., 2009) and in hypertension (Rudic and Fulton, 2009). In addition to CYP1A1 induction, PAHs in cigarette smoke are known to increase CYP1A2 expression (Schrenk et al., 1998; Caubet et al., 2002; Nebert and Russell, 2002) which has been shown to be involved in the hepatic metabolism of melatonin (Kall and Clausen, 1995; Facciolá et al., 2001; Härtter et al., 2001). As a result, CYP1A2 activity is elevated in smokers and decreases after smoking cessation. A recent study reported that plasma melatonin levels are significantly reduced in smokers (Ozguner et al., 2005), although another study reported that cigarette smoking lowered exogenous serum melatonin levels (following oral administration) but did not affect endogenous levels. Melatonin has been shown to possess anti-inflammatory effects and may reduce tissue damage during inflammatory responses via a number of mechanisms (Reiter et al., 2000). In addition to scavenging free radicals of reactive oxygen and nitrogen species, melatonin has been shown to prevent the nuclear translocation and DNA binding of NF-κB, thereby reducing the upregulation of various proinflammatory cytokines (Reiter et al., 2000). There is also evidence that melatonin inhibits the production of adhesion molecules and reduces leukocyte-endothelial adhesion and leukocyte transendothelial cell migration (Reiter et al., 2000). Thus, a reduction in melatonin levels is one example by which BaP and ETS could alter circadian blood pressure levels related to the inflammatory response.

In terms of peripheral circadian clocks, both endothelial function (Keskil et al., 1996) and vasomotor tone exhibit a circadian rhythm (Panza et al., 1991). Nitric oxide may be involved in regulating blood pressure rhythm (Witte et al., 1995) and increased oxidative

stress has been reported to disrupt the timing of circadian clock (Hardeland et al., 2003). However, while BaP or ETS-induced inflammatory reactions have the potential to cause oxidative stress and endothelial dysfunction, results from the BaP experiment indicate that altered NO or ET-1 levels do not explain the association between lung inflammation and increased blood pressure during sleep/inactivity. Thus, oxidative stress-mediated impairment of endothelial function does not appear to be the major mediator of altered blood pressure rhythm. Alterations in other vasoactive mediators, such as AngII and/or prostaglandin, may instead have played a role in the ETS and BaP-induced alteration in blood pressure rhythm.

5.5 Overall Conclusions

This study has shown that the PAH component of cigarette smoke may be responsible for the ETS-induced increase in blood pressure and the loss of dipping pattern during sleep. This BaP- and ETS-induced effect was associated with increased neutrophil recruitment into the lung, suggesting a possible link between altered circadian blood pressure rhythms and inflammation. Subchronic ETS exposure also resulted in increased arterial stiffness, likely as a result oxidative stress and reduced NO bioactivity. ETS-induced CYP1A1 activity and subsequent formation of reactive metabolites in the lung may indirectly be responsible for the increases in systemic oxidative stress. This may occur via release of inflammatory mediators from the lungs into the bloodstream which then cause vascular oxidative stress and subsequently affect vascular function. Since BaP exposure caused similar blood pressure effects as ETS, but did not alter CYP1A1 activity, NO bioactivity or arterial stiffness, factors

other than oxidative stress-mediated endothelial dysfunction may be responsible for the loss of blood pressure dipping during sleep. Finally, results from this thesis demonstrate that pulse wave dP/dt can be used as an indicator of arterial stiffness and highlights the importance of assessing circadian patterns in blood pressure via blood pressure telemetry in experimental animals. The next logical choice for experiments would be to compare the cardiovascular effects of ETS and BaP exposure to that of other environmental sources of PAHs, such as air pollution or diesel exhaust particles.

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